CD63 as an Activation-Linked T Cell Costimulatory Element

Katharina Pfistershammer, Otto Majdic, Johannes Stöckl, Gerhard Zlabinger, Stefanie Kirchberger, Peter Steinberger and Walter Knapp

*J Immunol* 2004; 173:6000-6008; doi: 10.4049/jimmunol.173.10.6000

http://www.jimmunol.org/content/173/10/6000

**References**

This article cites 58 articles, 31 of which you can access for free at: http://www.jimmunol.org/content/173/10/6000.full#ref-list-1

**Subscription**

Information about subscribing to *The Journal of Immunology* is online at: http://jimmunol.org/subscription

**Permissions**

Submit copyright permission requests at: http://www.aai.org/About/Publications/JI/copyright.html

**Email Alerts**

Receive free email-alerts when new articles cite this article. Sign up at: http://jimmunol.org/alerts
CD63 as an Activation-Linked T Cell Costimulatory Element

Katharina Pfistershammer, Otto Majdic, Johannes Stöckl, Gerhard Zlabinger, Stefanie Kirchberger, Peter Steinberger, and Walter Knapp

Dendritic cells (DC) are unique in their capacity to either stimulate or regulate T cells, and receptor/ligand pairs on DC and T cells are critically involved in this process. In this study we present such a molecule, which was discovered by us when analyzing the functional effects of an anti-DC mAb. This mAb, 11C9, reacted strongly with DC, but only minimally with lymphocytes. In MLR it constantly reduced DC-induced T cell activation. Therefore, we assumed that mAb 11C9 primarily exerts its functions by binding to a DC-structure. This does not seem to be the case, however. Preincubation of DC with mAb 11C9 before adding T cells had no inhibitory effect on T cell responses. Retroviral expression cloning identified the 11C9 Ag as CD63. This lysosomal-associated membrane protein (LAMP-3), is only minimally expressed on resting T cells but can, as we show, quickly shift to the surface upon stimulation. Cross-linkage of that structure together with TCR-triggering induces strong T cell activation. CD63 on T cells thus represents an alternative target for mAb 11C9 with its binding to activated T cells rather than DC being responsible for the observed functional effects. This efficient CD63-mediated costimulation of T cells is characterized by pronounced induction of proliferation, strong IL-2 production and compared with CD28 enhanced T cell responsiveness to restimulation. Particularly in this latter quality CD63 clearly surpasses several other CD28-independent costimulatory pathways previously described. CD63 thus represents an activation-induced reinforcing element, whose triggering promotes sustained and efficient T cell activation and expansion. The Journal of Immunology, 2004, 173: 6000–6008.

It is generally accepted that efficient activation of resting T cells requires a secondary signal in addition to signal 1 that is normally provided via specific engagement of the TCR complex with a defined peptide-MHC complex (1–3). This signal 2 is generally referred to as costimulation and significantly lowers the number of TCR complexes needed to be triggered for the induction of activation and the acceleration of T cell responses (4). Characteristic features of an effective costimulation process are induction of high levels of IL-2, prevention of anergy, and enhanced cell survival (5). The primary costimulatory pathway in T cells is the ligation of CD28 on T cells with B7.1/2 on APC (6). Still, mice deficient in CD28 are able to raise immune responses, indicating that molecules distinct from CD28 are also able to exert similar effects (7). Costimulatory function has been attributed to a number of molecules. Among them, molecules that prolong and favor T cell-APC contact are well established to act in a costimulatory fashion. Two main structures on T cells involved in maintenance of this cellular interaction are CD2 and LFA-1 (8, 9). Activation of LFA-1, a key structure of the immunological synapse, is one of the first consequences of a T cell signal (10, 11). Other well-established costimulatory molecules like OX40, CD27, and 4-1BB belong to the TNFR family (6, 8). A number of non-CD28 costimulators, like CD5, CD44, and CD9 have been reported to exert their effects by favoring the association of TCR complexes with lipid rafts (12). In addition, CD81 and CD82, like CD9 members of the tetraspan family, have been reported to be involved in T cell activation processes (13, 14). CD63 is also a member of this family and was up to now mainly described as a marker molecule for lysosomal-associated membrane protein (LAMP)3 compartments (also termed LAMP-3) (15). It was studied for its role in cellular spreading (16) and in the pathogenesis of melanoma (17, 18). Furthermore, CD63 is known as a marker of activation of several cell types including granulocytes and platelets (19–22). Of particular importance clinically has become the analysis of CD63 expression on human basophils, which significantly increases upon their activation (23).

In contrast, there are only a few studies concerning the expression of CD63 on T cells and its regulation and function on these cells (24–28). We show that CD63 is induced on T cells upon activation and demonstrate that activation-induced T cell surface CD63 has a number of interesting functional effects. Although blocking CD63 on T cells with soluble CD63 mAb 11C9 impairs dendritic cell (DC)-induced T cell proliferation, cross-linking of CD63 via mAb 11C9 delivers a potent costimulatory signal to T cells. This CD63-based costimulatory signal differs from other CD28 independent costimulatory pathways in its high efficacy and furthermore increases the responsiveness of T cells to restimulation.

Materials and Methods

Media and reagents

Cells were cultured in RPMI 1640 medium (Invitrogen Life Technologies, Paisley, Scotland) supplemented with 2 mM L-glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin, and 10% FCS (Sigma-Aldrich, Vienna, Austria). Recombinant human GM-CSF and IL-4 were kindly provided by the Novartis Research Institute (Vienna, Austria). PHA, PMA, ionomycin, LPS (from Escherichia coli serotype O127-B8), and propidium iodide (PI) were obtained from Sigma-Aldrich Chemie (Deisenhofen, Germany). CD3/CD28 mAbs coated beads (Dynabeads CD3/CD28 T cell expander) were obtained from Dynal. Bovine serum albumin (BSA) and goat anti-mouse IgG or anti-rat IgG were purchased from Sigma Chemie (Deisenhofen, Germany). Antibodies were used as described (3). Anti-CD3 mAb (clone 145–2C11, Beckman Coulter, Krefeld, Germany) was used to obtain T cell suspension. Anti-CD28 mAb (clone 37.5.1, Pharmingen, San Diego, CA) was used to coat beads. Anti-CD63 mAb (clone 11C9) was purified from ascites fluid (15). mAb 11C9 was produced in our laboratory. Anti-TNF mAb (clone 5G2, kindly provided by Dr. H. M. Seib, South Germany, Germany) was used to block TNF activity. Mouse IgG1 was used as an isotype control. Anti-LAMP mAb (clone 11C9) was produced in our laboratory. Anti-LAMP mAb was affinity purified on a column of immobilized CD63. Human neutrophils were separated from peripheral blood by dextran sedimentation and adherence to plastic. Cells were stimulated by LPS (from Escherichia coli) for 1 h, washed once, and incubated in the presence or absence of 1 μg/ml mAb 11C9 for 30 min at 4°C. After washing, cells were analyzed by flow cytometry (FACSCaliber, BD Biosciences, San Jose, CA).

Received for publication March 22, 2004. Accepted for publication August 31, 2004. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked advertisement in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

1 This work was supported by a grant from the Austrian Science Fund (SFB005.2).

2 Address correspondence and reprint requests to Dr. Walter Knapp, Institute of Immunology, Medical University of Vienna, Borschkegasse 8A, A-1090 Vienna, Austria. E-mail address: walter.knapp@meduniwien.ac.at

3 Abbreviations used in this paper: LAMP, lysosome-associated membrane protein; DC, dendritic cell; mDC, monocyte-derived DC; PI, propidium iodide.
were obtained from Dynal Biotech (Oslo, Norway). CFSE for proliferation analysis was purchased from Molecular Probes (Eugene, OR).

Antibodies

Monoclonal Abs 11C9 was obtained by immunizing BALB/c mice with human monocyte-derived DC (mDC) and selecting for mAbs, which bind to DC and interfere with DC-T cell interactions.

Fab of mAb 11C9 were prepared using the Avidchrom Fab kit (Unisyn Technologies, San Diego, CA) following the manufacturers recommendations. Preparations were purified by FPLC superdex gel filtration (Pharmacia, Uppsala, Sweden) and removal of intact IgG with protein A superdex. The quality of the Fab preparation was checked in binding assays with fluorochrome-labeled Fc- and Fab-specific Abs.

Other murine mAbs that were generated in our laboratory are VlAP (calf intestine alkaline phosphatase-specific) used as nonbinding control Ab, VIT10 (CD1a), VIM12 (CD11b), VIM13 (CD14), CD33-3D3, 1/47 (MHC class II), AAA1 (CD147), VIT4 (CD4), VIT8 (CD8), CD25-3G10, 1-45E (CD58), 5-272 (B7-H1), M80 (CD14), CD5-5D7, VIT12 (CD6), CD7-6B7, VIT14 (CD27), and 7-480 (CD80). The CD14 mAb MEM18 and the CD3 mAb UCHT-1 were kindly provided by An der Grub (Bio Forschung, Kaumberg, Austria) and the CD19 mAb (BU12) provided by An cell (Bayport, MN). The mAbs CD16-3G8, CD8 (S4.1), Tricolor, CD83 (BB15) and CD66 (BU63) were purchased from Caltag Laboratories (Burlington, CA). mAbs specific for human CD3, CD25, CD40, and CD63 (H6C5) were from American Type Culture Collection (Manassas, VA) and mAbs to CD28 (Leu28), CD69 (HB15) and CD86 (BU63) were purchased from Caltag Laboratories (Burlington, CA). The CD14 mAb MEM18 and the CD3 mAb UCHT-1 were kindly provided by An der Grub (Bio Forschung, Kaumberg, Austria) and the CD19 mAb (BU12) provided by An cell (Bayport, MN).}

Fluorescence staining

For flow cytometric analysis, cells (5 x 10^6/ml) were incubated with fluorochrome-conjugated mAbs or unlabeled primary Ab (10 μg/ml) for 20 min on ice and washed. For indirect staining, Oregon green conjugated anti-mouse Ig (Molecular Probes) was used as a secondary reagent. Staining of Fc receptor-bearing cells was done in the presence of human IgG Abs (20 mg/ml, Beriglobin; Aventis Behring, Vienna, Austria). For double staining or labeling of Fc receptor-bearing cells was done in the presence of human IgG anti-mouse Ig (Molecular Probes) was used as a secondary reagent. Staining was performed using a FACSCalibur flow cytometer (BD Immunocytometry Systems, San Jose, CA) supported by CellQuest software (BD Biosciences, Palo Alto, CA). For stimulation of T cells the CD3 mAb OKT3 (Ortho Pharmaceutical, Raritan, NJ) was used.

Cell preparations

Mononuclear cells from peripheral blood were isolated from heparinized whole blood of healthy donors by standard density gradient centrifugation with Ficoll-Paque (Pharmacia). Monocytes and T cells were separated by PBMC by magnetic sorting as previously described (29). Monocytes were enriched by using biotinylated CD14 mAbs VIT10 and MEM18 (purity >95%). Purified T cells were obtained through negative depletion of CD11b, CD14, CD16, CD19, CD33, and MHC class II-positive cells with the respective mAbs (purity >95%).

DCs were generated by culturing CD14+ monocytes in the presence of GM-CSF (50 ng/ml) and IL-4 (100 U/ml) for 7 days (29). For the generation of mature DC, LPS (1 μg/ml) was added to DC cultures on day 6 of cultivation. Activated T cells were generated by culturing PBMC (1 x 10^6/ml) up to 3 days in the presence of soluble PHA (5 μg/ml), plate-bound CD3/CD28 mAbs (1 μg/ml and 2 μg/ml) or allogenic DC (ratio 1:10).

Generation of the BwCD64, BwCD64/CD80, and the BwCD64/CD63 cell line

The generation of the murine thymoma cells Bw5147 (referred to as Bw cells throughout this work) expressing CD64 or CD64/CD80 is described elsewhere (30). To generate the BwCD64/CD63 cell line BwCD64 cells were retrovirally transduced with the human CD63 cDNA. From the cell pool, CD63+ cells were selected by MACS to obtain the BwCD64/CD63 cell line.

T cell proliferation assays

For T cell proliferation assays, highly purified T cells were cultured with the respective stimuli in 96-well cell culture plates (Corning; Costar, Vienna, Austria). For the last 18 h [methyl-3H]Tdr (ICN Pharmaceuticals, Irvine, CA) was added. Cells were harvested and incorporated [methyl-3H]Tdr was measured on a microplate scintillation counter (Packard; Topcount Instrument, Meriden, CT). All T cell proliferation assays were performed in triplicate.

For the primary MLR, allogenic T cells (1 x 10^5/well) were cocultured with graded numbers of DC for 5 days in presence of mAbs at a final concentration of 10 μg/ml.

For stimulation with Ab-coated beads T cells (1 x 10^5/well) were cultured with graded numbers of Dynabeads T cell expander for 4 days in presence of mAbs at a final concentration of 10 μg/ml.

For T cell stimulation with the Bw cell lines, BwCD64/CD63, BwCD64/CD80, and the control BwCD64 cells were irradiated (6000 rad) and incubated with purified anti-CD3 mAb (final concentration 10 ng/ml) or with mAbs to CD3 and CD28 (final concentration, 10 ng/ml each) and added to flat-bottom 96-well plates (2.5 x 10^4 cells per well). Purified human T cells (5 x 10^5) were added to each well and cocultivated for 3 days.

For T cell stimulation with plate-bound Abs, 96-well flat-bottom plates were incubated over night at 4°C with either CD3 (1 μg/ml) alone or in combination with CD28, CD63, or CD47 Abs (2 μg/ml). Plates were washed and blocked with medium containing FCS for 4 h at room temperature. Purified human T cells (5 x 10^5) were added and T cell proliferation was measured after 96 h of culture. For cytokine measurement culture supernatants were harvested after 72 h and cytokines were measured as described (31).

In restimulation experiments, 1 x 10^5 purified T cells per well were cultured for 7 days in presence of the following combinations of plate-bound mAbs: CD3/CD147, CD3/CD28, or CD3/CD63. After 7 days of primary culture, cultured T cells were harvested and restimulated using either plate-bound mAbs (CD3/CD28 or CD3/CD63), or different types of allogeneic APC (immature DC, mature DC, or monocytes, ratio 1:5) for 2 days.

Measurement of number of cell cycles by CFSE staining

CFSE labeling was performed as previously described (32). Briefly, T cells (5 x 10^5/ml) resuspended in PBS were incubated with 5 μM CFSE for 4 min at room temperature. The staining process was stopped by addition of medium. Cells were washed, resuspended in medium, and used for proliferation assays as described. At the indicated time point cells were harvested and washed in PBS and analyzed by flow cytometry. Dead cells were excluded by using PI. To assess proliferation of the CD4+ and the CD8+ subset, cells were counterstained with Abs to these molecules.

Assessment of apoptosis

T cells were incubated with immobilized mAb in 96-well at 37°C for 3 or 7 days. Apoptosis was assessed by staining with FITC-labeled annexin V (Caltag Laboratories) and PI and flow cytometric analysis. Annexin V-positive and PI-negative cells were scored as apoptotic cells.

RT-PCR analysis of Bcl-2 and Bcl-xL expression

T cells were stimulated with immobilized mAb for 72 h and used for RNA preparation. RNA (1 μg) was used for cDNAs synthesis using oligo(dT) priming. cDNAs were amplified with primers specific for Bcl-2 (5’-CTCTT CAGGGACGGGGTGAA-3’ and 5’-TGGATCCAGGGTGTTGAG-3’), and Bcl-xL (5’-CTGAGGGCCCCAAGGAGACT-3’ and 5’-GTCCTG GGTGATTGTTGAG-3’), and control primers specific for G3DPH (5’-TCAGGACCGCTTGGCAACTCA-3’ and 5’-AGGGGAGATCAA GTTGGTGTG-3’).

Retroviral expression cloning

cDNA cloning was performed as described by us before (30). Briefly, a retroviral cDNA library derived from immature and mature mDC was expressed in Bw cells. Cells expressing the 11C9 Ag were repeatedly enriched by MACS. Single cell cultures were obtained by limiting dilution culture and genomic DNA was prepared from cell clones expressing the 11C9 Ag. The cDNA insertions were PCR amplified from genomic DNA with primers specific for the flanking retroviral sequences using the Expand PCR system (Roche Applied Sciences, Mannheim, Germany). The obtained PCR products were gel-purified and cloned using Topo cloning (Invitrogen Life Technologies). Selected plasmids were transfected into 293 T cells using Lipofectamine to confirm that mAb 11C9 reacts with the protein identified by the isolated cDNA.

Statistics

Mann-Whitney U test was used to assess significances. Differences were considered significant at p < 0.05.
Results
Monoclonal Ab 11C9 inhibits DC-induced T cell proliferation

The aim of this study was to identify immunoregulatory cell surface structures involved in DC-T cell interaction. For this purpose mAbs were tested for their capacity to modulate the stimulatory function of DC. mAb 11C9 fulfilled these criteria. Its addition to MLR at culture onset consistently reduced the proliferative response of T cells to allogenic DC (Fig. 1). The mean inhibitory effect obtained by addition of mAb 11C9 was 43% ± 15% (p < 0.05; n = 4). mAb 11C9 exerted its effect independently of the type of APC used because it was also observed with mature DC, which are potent T cell activators, and with monocytes, which only induce weak T cell stimulation (data not shown).

Monoclonal Ab 11C9 recognizes the CD63 Ag

The observed functional capacity of mAb 11C9 prompted us to identify the Ag recognized by this mAb. For this purpose we screened a retroviral expression library from mdDC (30) with our mAb. Transduced cells expressing the 11C9 Ag were isolated by MACS and used for preparation of genomic DNA. A PCR product (0.9 Kb) obtained from a single cell clone (Fig. 2A) was introduced into an eukaryotic expression vector, and 293 T cells transfected with this construct were selectively reacting with mAb 11C9 (Fig. 2B). The nucleotide sequence of our 11C9 cDNA clone was found to be identical with the sequence of CD63 (GenBank accession number GI33876593). CD63 is a member of the tetraspan family, also known as LAMP-3, LIMP, lysosome-integrated membrane protein, or melanoma-associated Ag.

CD63 has previously been described as a marker of the myeloid cell lineage and is routinely used as an activation marker of basophil granulocytes (23). Lymphocytes have been reported to be CD63 negative (24). Using our CD63 mAb 11C9, we found the expected expression profile with intracellular and extracellular expression of monocytes and DC, intracellular expression in resting granulocytes, and surface expression of CD63 on granulocytes only upon activation. Regarding T lymphocytes, we identified a small subset (3–5%) of peripheral blood CD3+ T cells that displays CD63 on the cell surface. Strong intracellular reactivity with our mAb was found in the majority of the CD3+ T cells (Fig. 3A). However, upon activation with PHA, mAbs against CD3, CD25, and CD28, or allogenic APC most T cells were induced to express large amounts of cell surface CD63 (Fig. 3, A and B). Surface CD63 expression was induced to a similar extend on CD4+ and CD8+ T cells (Fig. 3C). The kinetics of this activation-induced surface expression of CD63 are fast. Expression can be detected after 5 h, similar to the established and widely used early activation marker CD69, although the expression level of CD63 is lower. Activation-induced CD63 expression increases up to 72 h whereas CD69 expression already declines by this time point (Fig. 3D).

Interestingly when further analyzing the population of CD63+ peripheral T cells in triple staining experiments, we found that these cells were distinct from CD69+ and CD25+ or MHC class II+ T cells (Fig. 3E). These cells thus characterize a population that differs from activated T cells.

CD63 surface expression on APC has no influence on their T cell stimulatory capacity

Because our results showed that CD63 is not only found on DC but also strongly expressed on activated T cells, the inhibitory effect of mAb 11C9 could either result from binding to T cells or DC. To clarify this, we first performed a number of experiments to test whether interaction of mAb 11C9 with CD63 on DC is responsible for the reduced proliferative response of T cells in an allo-MLR. First we tested whether interaction of mAb 11C9 with CD63 has an effect on the in vitro differentiation or maturation of mdDC. Presence of our mAb did not interfere with these processes as we could not see any alteration in the expression profile of markers for DC differentiation and maturation (Fig. 4, A and B).

In addition a similar inhibitory effect of DC-induced T cell activation was seen when adding Fab of 11C9 instead of whole Ab to an allo-MLR further excluding the possibility that a signal delivered by CD63 mAb to DC is responsible for the observed effects (data not shown). In additional experiments we pretreated DC with CD63 mAb 11C9 or a control mAb (M80), removed nonbound Ab
by washing and added the DC to allogenic T cells. Under these conditions, mAb 11C9 had no inhibitory effect on T cell stimulation (Fig. 4C).

To study the effects of CD63 surface expression directly we used artificial APC. We used stable transfectants of the murine thymoma cell line Bw expressing the human high affinity Fc receptor CD64 (BwCD64). These cells were further transduced to coexpress either CD63 (BwCD64/CD63) or as a control human CD80 (BwCD64/CD80; Fig. 5A). All transductants can be efficiently loaded with mAbs via the high affinity FcγR CD64 (30). BwCD64/CD63 cells, like BwCD64 cells loaded with CD3 mAb alone, cannot induce highly purified T cells to proliferate, indicating that CD63 on APC is not able to costimulate the activation of the TCR complex (Fig. 5B). In contrast, BwCD64/CD80 induced strong T cell proliferation when loaded with CD3 mAb alone. In the presence of CD3 and CD28 mAbs, all three cell types were able to induce T cell proliferation. Expression of CD63 on the artificial APC, however, had no influence on T cell stimulation mediated by cross-linked CD3 and CD28 mAbs (Fig. 5B).

Taken together our results indicate that CD63 on APC does not act in a costimulatory manner and that the effects observed with mAb 11C9 are not due to the delivery of a negative signal to DC. Therefore it is likely that mAb 11C9 exerts its inhibitory effect by interacting with CD63 on T cells. Because Fab of 11C9 has a similar effect as whole Ab (data not shown) transduction of a negative signal to T cells by our Ab is improbable. Interestingly, presence of CD3 mAb 11C9 did not affect T cell activation mediated by CD3/CD28 mAb coated beads (data not shown). Thus it is likely that mAb 11C9 blocks the interaction of a costimulatory DC structure with CD63 on T cells.

Cross-linking of CD63 provides a costimulatory signal to T cells

To investigate whether a costimulatory signal can be delivered to T cells via CD63, the effects of plate bound mAb 11C9 alone or in combination with other mAbs were analyzed. Purified T cells were cultivated with plate-bound CD3 mAb alone or in combination with CD28 mAb, CD63 mAb, or a binding control mAb (AAA1). CD3 mAb alone only led to a very weak T cell proliferation as did the combination of CD3/control mAb. The slightly reduced T cell proliferation obtained with CD3/control mAb compared with CD3 mAb alone is probably due to a reduced amount of immobilized CD3 mAb in the presence of a second mAb. Stimulation with CD3

---

**FIGURE 3.** Expression of CD63. A. The surface and total (surface + intracellular) expression profile of CD63 was analyzed by flow cytometric analysis of different cell preparations (filled histograms). A control mAb (VIAP) was used in all experiments (open histograms). B. CD63 surface expression on T cells is inducible via TCR triggering. T cells were activated via plate-bound mAb to CD3 and CD28 or by cocultivation with allogenic APC. Surface reactivity with mAb 11C9 of activated (filled histograms) and resting T cells (bold line) and control staining with VIAP (dotted line) are shown. C. CD63 surface expression was analyzed on the CD4+ and the CD8+ subset of T cells activated with CD3/CD28 immobilized mAb. D. Kinetic of the CD69 and CD63 surface expression on T cells. MNC were stimulated with PHA and CD69 or CD63 surface expression of T cells (CD3+) at different time points are shown. E. Fresh PBMNC were analyzed. T cells (CD3+) were measured for coexpression of CD63 with early and late activation markers CD69, CD25, and MHC class II.
mAb together with CD28 mAb led to strong T cell proliferation, but, surprisingly, the use of immobilized CD63 mAb in combination with CD3 mAb induced proliferative rates in T cells that were as strong. CD63 mAb alone did not induce any T cell proliferation (Fig. 6A). Plate-bound CD63 mAb was also able to further enhance CD28-costimulated T cell proliferation in some but not all experiments and could induce activation of T cells in combination with low amounts of CD3/CD28 mAbs that when used alone did not show any effect (data not shown).

T cell stimulation with immobilized CD3 and CD63 mAbs did not only result in strong proliferation but also in comparable or even higher induction of the activation markers CD69 and CD25 on T cells (Fig. 6B). Interestingly, in contrast to CD28 mAb, soluble CD63 mAb was not able to enhance CD3-triggered T cell proliferation (data not shown). As solid phase immobilization to the plate surface is required, strong cross-linking seems to be necessary for costimulation via CD63.

To further characterize CD63 costimulation, the kinetics of T cell proliferation were studied with the CFSE labeling technique. Compared with CD28 costimulation, the number of T cells that did not proliferate was slightly higher. However, the majority of CD63 costimulated cells did enter cell cycling and underwent comparable numbers of cell cycles during 7 days as CD28 costimulated cells (Fig. 6C). At earlier time points we could, however, notice a small delay in the onset of cell cycling in T cells costimulated via CD63 (data not shown). This could be explained by the fact that CD63 needs to be induced before stimulation. Similar to costimulation via CD28, costimulation via CD63 led to proliferation in both the CD4^+ and the CD8^+ subset (Fig. 6C). Purified CD8^+ T cells could be also very efficiently activated via immobilized mAb to CD3 and CD63 (data not shown) indicating that costimulation of CD8^+ T cells via CD63 does not require CD4^+ T cell help.

Costimulation via CD63 induces IL-2 production in T cells

The induction of sufficient and prolonged IL-2 production is necessary to generate activated T cells that are fully functional. CD28

FIGURE 4. The inhibitory effect of mAb 11C9 in an allo MLR is not due to its interaction with CD63 on DC. A. CD63 mAb 11C9 does not interfere with DC differentiation. Monocytes were cultivated with GM-CSF and IL-4 for 7 days in presence of mAb 11C9 or a control mAb. DCs were analyzed for expression of surface markers as indicated. B, CD63 mAb 11C9 does not interfere with DC maturation. Immature DC were matured with LPS in presence of either mAb 11C9 or a control mAb for 2 days. Cells were analyzed for expression of maturation markers. C, Preincubation of DC with mAb 11C9 has no effect on their allostimulatory capacity. Purified T cells were cultured with graded numbers of allogenic DC preincubated over night with mAb 11C9 or control Ab M80.

FIGURE 5. Presence of CD63 on artificial APC has no influence on their capacity to stimulate T cells. A, FACS analysis of BwCD64, BwCD64/CD80, and BwCD64/CD63 cells using mAb specific for CD64, CD80, and CD63 (gray histograms). Reactivity of the control mAb (open histograms) is shown. B, Irradiated BwCD64, BwCD64/CD80, and BwCD64/CD63 cells were loaded with Abs as indicated and cocultured with purified human T cells. Data of one experiment are representative of three independent experiments shown.
CD3/CD28 and CD3/CD63 mAb was found. IFN-γ production was measured by ELISA. Data of one experiment representative for four independent experiments are shown. The differences between the production induced with CD3 mAb alone and in combination with CD63 or CD28 mAb were statistically significant \( p < 0.01; n = 5 \). This enhanced responsiveness of CD63 costimulation leads to induction of survival genes T cells fully activated by signal 1 and signal 2 up-regulate anti-apoptotic pathways. Many of the CD28 independent costimulatory pathways, however, have been reported to induce brief T cell activation followed by apoptosis \( (5, 35) \). We therefore compared T cells costimulated via CD28 with CD63 in this aspect.

We performed annexin V staining after 3 or 7 days of T cell activation with plate bound mAbs. No signs of enhanced apoptosis of CD3/CD63 mAbs stimulated T cells compared with CD3/CD28 mAbs stimulated T cells could be detected even after prolonged cultivation \( \text{Fig. 7A} \). Furthermore, induction of the survival genes Bcl-2 and Bcl-x\(_L\) was comparable in T cells costimulated via CD28 and CD63 after 72 h of stimulation \( \text{Fig. 7B} \). We could however notice a delayed induction of survival genes when measuring Bcl-2 and Bcl-x\(_L\) expression at earlier time points \( \text{data not shown} \). This is in line with a delayed onset of proliferation that was observed in T cells upon CD63 costimulation compared with T cells costimulated via CD28.

**CD63 costimulation leads to induction of survival genes**

T cells costimulated via CD63 show enhanced responsiveness to restimulation

Many of the CD28-independent costimulatory pathways have been reported to induce only brief T cell activation that is followed by unresponsiveness \( (5, 35) \). We therefore compared T cells costimulated via CD63 with CD63 regarding their capacity to respond to secondary stimulation.

For that purpose, purified T cells were cultivated in presence of different immobilized mAbs. After 7 days the cells were harvested and restimulated with CD3/CD28 or CD3/CD63 mAbs for another 48 h. To control viability, we also restimulated the T cells with PMA/ionomycin. Although T cells prestimulated with medium or CD3 mAb alone showed no or only very weak responsiveness to restimulation, T cells activated during the first round of stimulation via triggering CD3 and either of the two costimulatory molecules fully responded to secondary stimulation \( \text{Fig. 8A} \). Furthermore, cells activated in primary cultures via CD3/CD63 even responded in the second stimulation better than CD3/CD28 primed cells \( p < 0.01; n = 6 \).

We tested next whether this enhanced responsiveness of CD63 costimulated T cells is also found when restimulating these cells with APC. Also in these experiments, we measured higher proliferative response of T cells costimulated via CD63 in the primary stimulation \( p < 0.05; n = 5 \) \( \text{Fig. 8B} \). This enhanced responsiveness of T cells preactivated via CD3 and CD63 was obtained regardless whether immature DC, mature DC, or monocytes were used to restimulate T cells.

Our results indicate that T cells can receive a strong costimulatory signal via CD63. In many aspects these T cells show no
functional differences to T cells activated via the primary costimulatory molecule CD28. They have however a higher capacity to respond to secondary stimulation.

Discussion

During T cell activation costimulation is an essential process, influencing the outcome of the immune response: In presence of costimulatory signals T cells become efficiently activated, whereas absence of costimulation leads to anergy (1–3).

We describe in this study CD63 as a novel potent, activation-induced costimulatory structure on T cells. We show that although only a small percentage of resting T cells displays CD63 on the surface, this molecule is quickly induced upon T cell activation in vitro. Activation via TCR triggering alone is sufficient to up-regulate CD63 surface expression. Targeting these CD63 molecules on T cells has functional implications: Binding of intact mAb 11C9 or its Fab to CD63 on T cells consistently inhibits their responses to allogenic mdDC, whereas cross-linking of CD63 with mAb 11C9 can costimulate the activation of the TCR complex in human T cells. These findings lead to the conclusion that CD63 might be involved in the delivery of a costimulatory signal.

A yet unidentified structure expressed on APC seems to be responsible for this activation via CD63, as blocking of CD63 by mAb 11C9 inhibits the T cell response to DC, but not to CD3/CD28 mAb-coated beads. Cross-linking of CD63 via immobilized mAb might therefore act in an agonistic manner for this DC struc-

ture. This effect seems, however, to require interaction with a specific epitope on CD63 as the commercially available CD63 Ab H6C5, while also inhibiting DC induced T cell responses, is not able to act in a costimulatory fashion with CD3 mAb when presented in solid phase bound form (data not shown).

There are at least two possibilities how CD63 could be involved in the transfer of a costimulatory signal from DC to T cells. First, CD63 could be the receptor of a costimulatory signal provided by DC. Although no ligand for CD63 has been described so far, other members of the tetraspan family have recently been reported to be able to act as receptors (36, 37). Therefore it is conceivable that CD63 directly interacts with a yet unidentified ligand on DC. In contrast, tetraspans are well known for their multiple cis-interactions and associations that build up a so called tetraspan network (38–43). Thus binding of mAb 11C9 might alter the interaction of CD63 with a molecule that is a critical receptor for a costimulatory signal delivered by DC. The involvement of a CD63-associated molecule could also explain the requirement for strong cross-linking, achieved by immobilizing mAb 11C9 to plastic surfaces, to induce CD63 triggered costimulation in T cells.

We show for the first time that CD63 has a costimulatory function on T cells. It shares this capacity with a number of other molecules aside from CD28, which is generally considered the primary costimulatory molecule on T cells (6, 44). Among these molecules are several members of the tetraspan family (6, 13, 14, 45–50). Costimulation via molecules distinct from CD28 seems in most instances not to be sufficient for inducing full T cell activation (6, 33). This was also seen with costimulation via CD9, the
tetraspan molecule most widely examined in this context (35). The main deficiency in this CD28-independent costimulatory pathway appears to lie in the induction of IL-2, resulting in insufficient antiangiogenic signals, which leads to only brief activation of T cells followed by apoptosis (5, 33–35, 51–53). We did not, however, notice marked differences regarding T cell proliferation, up-regulation of activation markers, and, most importantly, IL-2 production when comparing T cells costimulated via CD28 and via CD63. Furthermore, we did not observe enhanced apoptosis induced by CD63 costimulation and we found comparable induction of the survival genes Bcl-2 and Bcl-xL, in both costimulatory pathways. These results clearly set CD63 apart from other tetraspan molecules with costimulatory properties, but also from other non-CD28 molecules that were described to be able to costimulate T cell activation. Experiments in which we compared the costimulatory capacity of immobilized mAb with other inducible costimulatory molecules (CD9, CD40 ligand, CD134/OX40) with CD63 mAb 11C9 confirmed the unique capacity of this Ab to provide a potent costimulatory signal leading to sustained T cell activation (data not shown). A possible explanation of the CD28-like function of CD63 might be the PI3K binding motif, TXXM, expressed on the short cytoplasmic tail. This motif can be found in several molecules important for the fate of activated T cells such as CD28, ICOS and TRIM (54) but not in other tetraspan molecules described to be involved in T cell activation or in LFA-1, CD2, CD27, OX40, 4-1BB, CD40 ligand, CD5 or CD44. Furthermore, CD63-mediated costimulation is inhibited by the PI3K inhibitor Wortmannin in a similar manner as CD28 costimulation (data not shown). Aside from this CD28-like function of CD63 in primary activation we found it striking that T cells stimulated via CD3/CD63 mAbs showed even stronger responses upon restimulation than CD28-costimulated T cells. T cell activation is tightly controlled by surface and cytoplasmatic molecules to conserve T cell homeostasis (55–58). We found that costimulation of T cells via CD63 led to enhanced responsiveness regardless whether these cells were re-stimulated with immobilized Abs, allogenic APC, or mitogens. Thus it seems more likely that this feature of T cells costimulated via CD63 is due to altered regulation of components of their intracellular signaling machinery than due to differential expression or activation of stimulatory or inhibitory surface molecules. In agreement with that, we did not detect any evidence for changes in the expression profile of a number of surface molecules involved in costimulation in those T cells (data not shown).

Summarizing our results, we suggest that CD63 as an activation-induced costimulatory structure is involved in a reinforcing system: CD63, stored in the cytoplasma in resting T cells, is translocated to the plasma membrane upon incipient activation. It allows APC to deliver a potent costimulatory signal to T cells that further sustains and amplifies the ongoing activation, resulting in efficiently stimulated T cells. In the light of evidence for a CD63-dependent costimulatory pathway presented in this study, CD63 surface expression in a small subset of peripheral T cells might have functional consequences. Because we provide evidence that T cells can receive a strong costimulatory signal from APC via CD63, it is tempting to speculate that in vivo these cells might act in first line of defense upon antigenic challenge. We are therefore trying to phenotypically and functionally characterize these CD633+ peripheral T cells in current studies.

Acknowledgments
We thank Dr. David Segal for critically reading the manuscript and appreciate the excellent technical assistance of Petra Kohl, Alessandra Mathe, Margarethe Merio, Christoph Klauser, and Claus Wenhardt. We also thank Dr. Garry P. Nolan and colleagues for providing the retroviral vector pBMNZ and the packaging cell line Phoenix-E. Prof. Walter Knapp died August 30, 2004. We will always remember his enthusiasm for science and his mentorship.

References
9. Gagli, J. L., E. A. Greenfield, A. Matteo, A. H. Sharpe, G. J. Freeman, and V. F. Fuchsroo. 2000. Interleukin-15 expression in T cells is due to altered regulation of components of their intracellular signaling machinery than due to differential expression of stimulatory or inhibitory surface molecules. In agreement with that, we did not detect any evidence for changes in the expression profile of a number of surface molecules involved in costimulation in those T cells (data not shown).

Summarying our results, we suggest that CD63 as an activation-induced costimulatory structure is involved in a reinforcing system: CD63, stored in the cytoplasma in resting T cells, is translocated to the plasma membrane upon incipient activation. It allows APC to deliver a potent costimulatory signal to T cells that further sustains and amplifies the ongoing activation, resulting in efficiently stimulated T cells. In the light of evidence for a CD63-dependent costimulatory pathway presented in this study, CD63 surface expression in a small subset of peripheral T cells might have functional consequences. Because we provide evidence that T cells can receive a strong costimulatory signal from APC via CD63, it is tempting to speculate that in vivo these cells might act in first line of defense upon antigenic challenge. We are therefore trying to phenotypically and functionally characterize these CD633+ peripheral T cells in current studies.

Acknowledgments
We thank Dr. David Segal for critically reading the manuscript and appreciate the excellent technical assistance of Petra Kohl, Alessandra Mathe, Margarethe Merio, Christoph Klauser, and Claus Wenhardt. We also thank

References
9. Gagli, J. L., E. A. Greenfield, A. Matteo, A. H. Sharpe, G. J. Freeman, and V. F. Fuchsroo. 2000. Interleukin-15 expression in T cells is due to altered regulation of components of their intracellular signaling machinery than due to differential expression of stimulatory or inhibitory surface molecules. In agreement with that, we did not detect any evidence for changes in the expression profile of a number of surface molecules involved in costimulation in those T cells (data not shown).

Summarying our results, we suggest that CD63 as an activation-induced costimulatory structure is involved in a reinforcing system: CD63, stored in the cytoplasma in resting T cells, is translocated to the plasma membrane upon incipient activation. It allows APC to deliver a potent costimulatory signal to T cells that further sustains and amplifies the ongoing activation, resulting in efficiently stimulated T cells.

In the light of evidence for a CD63-dependent costimulatory pathway presented in this study, CD63 surface expression in a small subset of peripheral T cells might have functional consequences. Because we provide evidence that T cells can receive a strong costimulatory signal from APC via CD63, it is tempting to speculate that in vivo these cells might act in first line of defense upon antigenic challenge. We are therefore trying to phenotypically and functionally characterize these CD633+ peripheral T cells in current studies.


