Relevance of DC-SIGN in DC-induced T cell proliferation

Karijn Gijzen, Paul J. Tacken, Aukje Zimmerman, Ben Joosten, I. Jolanda M. de Vries, Carl G. Figdor, and Ruurd Torensma

Department of Tumor Immunology, Nijmegen Centre for Molecular Life Sciences, Radboud University Nijmegen Medical Centre, Nijmegen, The Netherlands

Abstract: The role of dendritic cell-specific ICAM-3-grabbing nonintegrin (DC-SIGN) in DC-T cell communication was assessed by analyzing the effect of DC-SIGN-blocking mAb in MLR. The results show that the degree of inhibition by DC-SIGN and LFA-1 mAb depends on the magnitude of the MLR and the maturation status of the DC. Addition of DC-SIGN mAb at several time-points during MLR showed that DC-SIGN is involved early on in DC-T cell contacts. This initial role is masked by strong adhesive and costimulatory mechanisms, indicating a short-lived effect of DC-SIGN in DC-T cell interactions. To examine this concept in more detail, the percentage of PBL capable of binding DC-SIGN was determined. Analysis of several donors revealed that 1–20% PBL bind to beads coated with recombinant DC-SIGN, and the DC-SIGN-binding cells comprised all major cell subsets found in blood. PBL isolated from a donor with high DC-SIGN-binding capacity were more prone to blocking by DC-SIGN mAb in MLR than PBL from a donor with low DC-SIGN-binding capacity. This study indicates an initial and transient role for DC-SIGN in T cell proliferation, which becomes apparent when T cell proliferation is low and when the percentage of DC-SIGN binding PBL is high. J. Leukoc. Biol. 81: 000–000; 2007.

Key Words: C-type lectins · integrins · mixed leukocyte reaction · conjugates · subsets

INTRODUCTION

Dendritic cells (DC) and T cells are important players of the immune system. Interactions between these two cell types can result in the induction of antigen-specific immunity or in tolerance [1]. DC are professional APC and reside in peripheral tissues on the alert for invading pathogens. After encountering and processing antigen, the DC will migrate to the secondary lymphoid organs to present peptides in MHC context to T cells [2]. During inflammation, DC mature and acquire properties to induce an effective immune response. In non-inflamed situations, however, DC remain immature and induce tolerance [1, 2]. In the lymph nodes, naïve T cells and DC interact transiently in an antigen-independent manner to enable the T cells to inspect a large number of MHC molecules on DC for the presence of a specific peptide [3]. ICAM-3 is an important adhesion molecule in these antigen-independent interactions between T cells and APC [4]. Binding partners for ICAM-3 include the β2-integrins LFA-1 [5] and αβ[6] and the C-type II lectin DC-specific ICAM-3-grabbing nonintegrin (DC-SIGN) [7]. This C-type II lectin is expressed in vitro on monocyte-derived DC and in vivo on DC in several tissues including lymph nodes, cervix, mucosa, and skin [7, 8]. In addition, DC-SIGN expression is detected on a subset of myeloid blood DC and on blood DC antigen 2$^+$ plasmacytoid DC precursors [8, 9]. As integrins need activation before appropriate binding can occur, DC-SIGN was put forward as an initial binding partner of ICAM-3 [7]. Upon specific peptide recognition by a T cell, the transient adhesive interactions between T cells and DC are strengthened, resulting in an immunological synapse [3]. This structure is formed by the interactions of several adhesion and costimulatory molecules such as LFA-1 interacting with ICAM-1 and/or ICAM-3 and CD28 interacting with CD80 and/or CD86 and provides a platform for sustained TCR engagement and signaling [10–12]. Such a mechanism predicts a transient role for DC-SIGN in DC-T cell interactions and may explain in part the conflicting data obtained in T cell activation studies so far [7, 13–16]. Studies of Geijtenbeek et al. [7] and Puig-Kroger et al. [13] showed that blocking DC-SIGN mAb have an inhibitory effect on the proliferation of resting T cells induced by allogeneic DC or THP-1 cells differentiated into DC. In contrast, Granelli-Piperno et al. [16] did not show a requirement of DC-SIGN in MLR driven by DC. Real et al. [14] could not confirm a role for DC-SIGN in early DC-T cell contact, as their studies did not reveal an effect of blocking DC-SIGN on immature DC on the CD4$^+$ T cell on CD69 expression, motility, and Ca$^{2+}$ response. However, Martinez et al. [15] analyzed early T cell activation as well by measuring IL-2 and IFN-γ secretion by CD4$^+$ T cells and showed that DC-SIGN can modulate this secretion dependent on the strength of the T cell stimulus. Moreover, these authors showed a modest down-regulation of CD69 expression on CD4$^+$ T cells in the presence of Chinese hamster ovary cells transfected with DC-SIGN. In the present study, numerous MLR, driven by immature or mature DC, were performed to unravel the relevance of DC-SIGN.
SIGN in MLR. We observed that blocking DC-SIGN only affects T cell proliferation when immature DC are used and when the proliferative response is weak. Likewise, inhibition of T cell proliferation by LFA-1-blocking mAb is also dependent on the strength of the T cell response. Moreover, the effect of blocking DC-SIGN in MLR depends on the percentage of PBL that are able to bind DC-SIGN.

MATERIALS AND METHODS

Cells

Immature DC were generated from human PBMC as described previously [7]. Briefly, PBMC were isolated from buffy coats of donated blood obtained from healthy individuals by Ficoll density centrifugation. Monocytes were isolated from PBMC by adherence to plastic and cultured in the presence of IL-4 and GM-CSF (500 and 800 U/ml, respectively, Schering-Plough, Brussels, Belgium) in RPMI-1640 medium (Gibco, Invitrogen, Breda, The Netherlands) containing 10% FCS (Greiner Bio-One B.V. Alphen aan den Rijn, The Netherlands) for 6 days. Nonadherent cells (PBL) were collected for later use. Mature DC were generated from immature DC by adding 2 µg/ml LPS (Sigma-Aldrich Chemie B.V. Zwijndrecht, The Netherlands) at Day 6. Mature DC were harvested on Day 7.

For testing DC-SIGN expression, DC were labeled in PBS containing 0.5% BSA and 0.01% sodium azide with mAb against DC-SIGN (5 µg/ml AZN-D1) or with isotype control mouse IgG1 (5 µg/ml, BD Biosciences, San Diego, CA) for 30 min at 4°C. After a washing step, DC were labeled by flow cytometry (FACS Calibur, BD Biosciences, Mountain View, CA).

HB2-2 cells were cultured in IMDM (Gibco, Invitrogen), supplemented with 5% FCS.

T cell proliferation assays

T cell proliferation was assessed by [3H]-thymidine incorporation or by CFSE methodology after 6 or 7 days, respectively. For the CFSE methodology, the PBL, resuspended at a density of 2 x 10^7 cells per ml in PBS, were labeled with 5 µM CFSE (Molecular Probes, Leiden, The Netherlands) for 10 min at room temperature (RT). Free CFSE was quenched by the addition of an equal volume of FCS. The labeled cells were washed twice in PBS and resuspended in RPMI-1640 medium containing 10% FCS [17]. PBL comprise mainly T cells, B cells, and NK cells. As those T and B cells are from the same donor, no MLR will be induced by DC-SIGN expressed by B cells [18]. MLR was induced by allogeneic DC. Unlabeled or CFSE-labeled PBL (1 x 10^6) were cocultured with 1.5 x 10^3 allogeneic DC in 96-well round-bottom plates for 6-7 days. At 0, 24, and 72 h after the start of the experiment, blocking mAb directed against DC-SIGN (10 µg/ml AZN-D1 and 10 µg/ml AZN-D3), LFA-1 (10 µg/ml NKI-L15), or CD6 (5 µg/ml, Clone M-T605, mouse IgG1, BD PharMingen, San Diego, CA) was added where indicated. Control mouse IgG1 (10 µg/ml, R&D Systems, Abingdon, UK) or total mouse IgG (10 µg/ml, Jackson ImmunoResearch, West Grove, PA) was included as controls. After 6-7 days, PBL proliferation was assessed by determining CFSE staining intensity by flow cytometry (FACS Calibur, BD Biosciences, Mountain View, CA) or by measuring [3H]-thymidine incorporation (1 µCi/well, 8-16 h pulse, MP Biomedicals Inc., Irvine, CA). To enable comparison of data obtained using different donors, proliferation data obtained under control conditions were expressed as 100% proliferation, and putative blocking was calculated relative to the control MLR for that given donor.

Conjugate formation assay

Allogeneic DC (10^5 cells) were mixed with 10^7 PBL and incubated for 15 min at 37°C while shaking. The DC-PBL mixture was allowed to adhere on poly-L-lysine (50 µg/ml)-coated glass slides for 5 min at RT. Next, cells were fixed with 2% paraformaldehyde in PBS at RT for 20 min. Nonspecific binding sites were blocked with blocking buffer (3% BSA, 10 mM glycine, and 1% human serum in PBS) at RT for 1 h. Cells were triple-stained with anti-DC-SIGN (DCN46, 10 µg/ml), anti-ICAM-3 (AZN-IC3, 10 µg/ml), and anti-CD3 (T3B, 10 µg/ml) in blocking buffer at RT for 1 h, followed by incubation with isotype-specific Alexa-488-, -568-, or -647-conjugated goat antimouse mAb for 1 h. Samples were analyzed using a MRC1024 confocal microscope (Bio-Rad, Hercules, CA) with a 60x objective.

DC-SIGN-His construct

Recombinant DC-SIGN-His consists of the extracellular domain of DC-SIGN harboring a six-His tag at the N terminus and was kindly provided by Dr. Clark (University of Washington, Seattle) [19]. Bacterial strain Escherichia coli M13(Prep) was transfected with this construct, and expression was induced by 0.1 mM isopropylthiogalactoside (Sigma-Aldrich Chemie B.V. Zwijndrecht). The protein was solubilized in 8.5 M urea and subsequently refolded by stepwise dialysis against buffers containing decreasing urea concentrations. The protein preparation was incubated with mannann agarse beads (Sigma-Aldrich Chemie B.V. Zwijndrecht). Only functional protein is able to bind to mannann agarse beads. After several washing steps to remove unbound protein, the functional DC-SIGN was eluted from the beads by EGTA, removing the essential Ca^2+ ion from DC-SIGN. After removal of the beads by centrifugation, the supernatant containing the functional protein was reconstituted with Ca^2+ ion containing buffer (20 mM Tris-HCl, pH 8.0, 150 mM NaCl, 1 mM CaCl_2, 2 mM MgCl_2, and 0.5% BSA). The six-His-tagged, humanized, single-chain hSG1.1 antibody was used as control and was kindly provided by Dr. Kretz-Rommel [20].

Binding assay with a soluble DC-SIGN-His construct

The soluble DC-SIGN-His construct (10 µg/ml) was incubated with 5 x 10^4 PBL in binding buffer (20 mM Tris-HCl, pH 8.0, 150 mM NaCl, 1 mM CaCl_2, 2 mM MgCl_2, and 0.5% BSA) in a 96-well V-shaped bottom plate at 37°C for 30 min. Where indicated, DC-SIGN-His constructs were preincubated with 100 µg/ml mannann (from Saccharomyces cerevisiae, Sigma-Aldrich Chemie B.V. Zwijndrecht) prior to the binding assay at RT for 20 min. Subsequently, the samples were incubated with mouse anti-Penta-His-Alexa-488 (Qiagen, Benelux B.V., Venlo, The Netherlands) at 37°C for 30 min. Cells were analyzed by flow cytometry, and the percentage of cells that had bound recombinant DC-SIGN-His was quantified.

Bead-binding assay with DC-SIGN-His construct

DC-SIGN-His or control-His were coated onto streptavidin-coated, carboxylate-modified TransFluoSpheres (488/645 nm, 1.0 µm, Molecular Probes [21]). First, streptavidin-coated beads were incubated with biotinylated horse antiamouse IgG (10 µg, Vector, Brunswich Chemieg, Amsterdam, The Netherlands) at 37°C for 2 h, followed by an overnight incubation with mouse anti-Penta-His (1 µg, Qiagen) at 4°C. Subsequently, the beads were incubated with 250 ng DC-SIGN-His or control-His at 4°C for 2 days.

The DC-SIGN and control beads were incubated with 5 x 10^4 PBL or HB2-2 cells in binding buffer (20 mM Tris-HCl, pH 8.0, 150 mM NaCl, 1 mM CaCl_2, 2 mM MgCl_2, and 0.5% BSA) in a 96-well V-shaped bottom plate at 37°C for 30 min. Where indicated, the beads were preincubated with 100 µg/ml mannann, 5 mM EGTA, or 100 µg/ml soluble ICAM-3 (Fc-ICAM-3 [7]) prior to the binding assay at RT for 20 min. In some experiments, PBL or HB2-2 cells were preincubated with 40 µg/ml ICAM-3-blocking mAb for 20 min at RT prior to the binding assay (C6B3/1 and #32 [7]). After washing, adhesion of the beads to the cells was assessed by flow cytometry.

Characterization of DC-SIGN-binding cells by flow cytometry

To characterize the DC-SIGN-binding cells, PBL were incubated with DC-SIGN-His-coated beads as described above. After washing, the cells were labeled for 20 min at RT with combinations of CD3-FTTC (DakoCytomation B.V., Heverlee, Belgium), CD3-PE (BD PharMingen), CD4-PE (BD PharMingen), CD8-FTTC (BD PharMingen), CD19-PE (BD PharMingen), CD25-PE (Becton Dickinson, San Jose, CA), CD45RA-PE (Immunotech, France), CD45RO-FTTC (DakoCytomation B.V.), CD65-PE (IQ Products, Groningen, The Netherlands), and/or CD62L-FTTC (DakoCytomation B.V.). Samples were analyzed by flow cytometry (Cytomics FlowJo 500, Beckman Coulter, Fullerton, CA).
RESULTS

DC maturation state affects contribution of DC-SIGN and LFA-1 in MLR

The role of DC-SIGN in T cell proliferation is controversial [7, 13–16]. To unravel the significance of DC-SIGN in T cell proliferation, numerous MLR were performed in the presence of DC-SIGN-blocking mAb, and mAb directed against LFA-1 was included as a positive control for blocking the MLR. Immature and mature allogeneic DC were used as T cell stimulators. T cell proliferation was determined by [3H]-thymidine incorporation or by CFSE dilution (Fig. 1A). Using both methodologies, a consistent block in T cell proliferation is observed when immature DC were used as stimulators. T cell proliferation was determined by [3H]-thymidine incorporation or by CFSE dilution (Fig. 1A and B), which yielded comparable results. As expected, with immature DC, a consistent block in T cell proliferation is observed when LFA-1 mAb is present (Fig. 1A). Using both methodologies, significant blocking was observed for LFA-1, as determined by ANOVA, followed by the Student Newman Keuls test: *P < 0.05. Taking the group as a whole, no significant effect of blocking DC-SIGN antibodies was observed in agreement with others [16]. However, in a subgroup of DC-T cell donor pairs, the presence of DC-SIGN mAb resulted in less T cell proliferation, as illustrated by their location outside the 99% confidence interval of control mouse IgG1. No correlation between expression profile of DC-SIGN on immature DC and the extent of the DC-SIGN effect on the T cell proliferation was observed (Fig. 1C).

When mature DC were used as stimulator cells (Fig. 1B), hardly any blocking effect is observed with DC-SIGN mAb. It is surprising that as LFA-1 is an effective mediator in MLR [22, 23], the blocking potential of the LFA-1 mAb is also decreased considerably when mature DC are used as T cell stimulators (Fig. 1, B and D). The blocking effect of DC-SIGN antibodies is observed when using immature DC as stimulator cells, and the blocking effect is absent when mature DC are used as stimulator cells (Fig. 1E) using the same donor for raising immature as well as mature DC and the same allogeneic PBL donor.

Together, these data show that the use of mature DC as T cell stimulators abrogates the contribution of DC-SIGN and LFA-1 in MLR.

DC-SIGN, LFA-1, and CD6 differentially influence DC-induced T cell proliferation over time

A full-blown T cell response, when measured at Day 7, results in a waning effect of DC-SIGN and LFA-1 mAb in MLR driven by mature DC. This could also hold for DC-SIGN in MLR driven by immature DC. Therefore, T cell proliferation was assessed after 3, 5, and 7 days. As positive control for blocking MLR driven by mature DC, a CD6-blocking mAb was included, as CD6 plays a crucial role in mature DC-T cell interactions [24]. In immature DC-T cell donor pairs, which could be blocked by DC-SIGN mAb, as depicted in Figure 1A, LFA-1, DC-SIGN, and CD6 mAb blocked T cell proliferation at all time-points indicated (Fig. 2A). However, T cell proliferation in DC-T cell donor pairs, which could not be blocked by DC-SIGN mAb when measured after 7 days, also did not show a blocking effect of DC-SIGN mAb when measured after 3 and 5 days (data not shown). It is remarkable in contrast to LFA-1 and DC-SIGN mAb that only the CD6 mAb was able to block proliferation induced by mature DC at all time-points indicated (Fig. 2B). LFA-1 mAb effectively blocked T cell proliferation induced by mature DC when measured after 3 days, but after 7 days, the blocking effect was hardly detectable. To further characterize the effect of blocking DC-SIGN during MLR, blocking mAb against DC-SIGN were added at 0, 24, or 72 h after onset of the immature or mature DC-T cell coculture (Fig. 2, C and D). The effects of blocking mAb to LFA-1 and CD6 were evaluated in parallel. In immature DC-T cell donor pairs, which could be blocked by DC-SIGN mAb, as depicted in Figure 1A, LFA-1 and DC-SIGN mAb blocked T cell proliferation effectively, only when added at the onset of the DC-T cell coculture (Fig. 2C). Similarly, LFA-1 mAb inhibited T cell proliferation induced by mature DC, only when added at the onset of the experiment (Fig. 2D). In contrast, CD6 mAb effectively blocked T cell proliferation induced by immature and mature DC when added at 0 and 24 h after onset of the experiment.

Altogether, these kinetic blocking experiments show that DC-SIGN and LFA-1 play a role early on in MLR, whereas CD6 mediates a long-lived effect.

Contribution of DC-SIGN and LFA-1 to MLR depends on strength of T cell response

In MLR, several T cells will be activated upon recognition of specific MHC peptide complexes expressed by the allogeneic DC. The number of T cells reacting to these MHC peptide complexes varies between different donors and consequently, the strength of the MLR response will vary. Indeed, in some MLR, a strong T cell-proliferative response was detected, whereas in others, the response was rather weak (Fig. 3A). This prompted us to separate the MLR into weak and strong responses based on the percentage of proliferating T cells as determined by CFSE staining. Based on several experiments, MLR, in which after 7 days, less than 60% of the T cells were detected in the proliferative pool, could be defined as a weak MLR, whereas MLR, in which more than 60% of the T cells were detected in the proliferative pool, could be defined as a strong MLR. When taking the group as a whole, no blocking effect of anti-DC-SIGN antibodies was observed. However, when categorized in this way, the effects of blocking DC-SIGN mAb were only visible in several weak MLR and when immature DC were used as stimulators (Fig. 3B). Similar to DC-SIGN mAb, LFA-1 mAb mostly affected MLR driven by immature DC, and the blocking effect was strongest in the weak MLR (Fig. 3C).

Thus, in strong MLR, proliferation cannot be blocked by DC-SIGN mAb and are blocked less efficiently by LFA-1 mAb.

Localization of DC-SIGN in DC-T cell contact area

To visualize the behavior of DC-SIGN in the DC-T cell contact area, we performed DC-T cell conjugate formation experi-
Fig. 1. DC maturation-dependent effects of DC-SIGN and LFA-1 on T cell proliferation. PBL (1 × 10^5) were cocultured with 1.5 × 10^5 immature (A) or mature (B) allogeneic DC. After 6–7 days, proliferation was assessed by [3H]-thymidine incorporation or by CFSE dilution. Control mouse IgG1 (mlgG1) or blocking mAb (10 μg/ml) directed against DC-SIGN (AZN-D1 and AZN-D3) or LFA-1 (NKI-L15) were added at the onset of the experiment. Data are percentages of control (no addition of mAb). Each dot represents the average of one independent experiment performed in triplicate. Each MLR experiment consists of a different allogeneic DC-PBL combination. Significant difference from mouse IgG control, as determined by ANOVA, followed by the Student Newman Keuls test: *, P < 0.05. Confidence interval (99%) of control mouse IgG1, assessed by [3H]-thymidine incorporation (immature DC, 86–161%; mature DC, 81–129%) and CFSE dilution (immature DC, 67–130%; mature DC, 63–112%), represented by gray area. (C) DC-SIGN (AZN-D1) expression on immature DC from two different donors. These DC were cocultured with different allogeneic PBL donors. Proliferation was assessed after 7 days (CFSE dilution). The percentage of proliferation with DC-SIGN mAb (relative to control) is depicted for each donor. Transparent histograms represent mouse IgG1 isotype control, and filled histograms represent DC-SIGN mAb. MFI, Mean fluorescence intensity. (D) Percentage of block in T cell proliferation mediated by LFA-1 mAb in MLR driven by allogeneic immature and mature DC. Data expressed as percentages of control (no addition of mAb) are mean ± SEM of 10 independent experiments, assessed by [3H]-thymidine incorporation or by CFSE dilution. Each MLR experiment consists of a different allogeneic DC-PBL combination. Significant difference from immature DC, as determined by unpaired two-tailed Student's t-test: *, P < 0.05. (E) CFSE profiles of T cells stimulated with immature or mature DC in the presence and absence of blocking antibodies. Proliferation was assessed after 7 days. In this experiment, the same allogeneic DC-PBL combination was used.
ments. Immature and mature DC formed contacts with T cells (Fig. 4). DC-SIGN is present in the DC-T cell contact area, although DC-SIGN is not recruited to the contact zone. No difference in distribution of DC-SIGN was observed between immature (Fig. 4A) and mature (Fig. 4B) DC-T cell conjugates. Participation of CD3+ cells in the DC-PBL conjugates or enrichment of ICAM-3 in the contact site did not influence the localization of DC-SIGN.

In conclusion, these data show that DC-SIGN is not enriched in the DC-T cell contact area.

Characterization of DC-SIGN binding to PBL

Various studies indicate that DC-SIGN-ICAM-3 interactions are of a transient nature [7, 25]. To examine this in more detail, we analyzed the binding capacity of PBL derived from various donors to a DC-SIGN-His construct used in free form or attached to streptavidin-coated fluorescent beads. DC-SIGN-His-coated beads showed the highest binding to PBL as compared with soluble DC-SIGN-His, with an average of 5.4% specific binding (Fig. 5A). It is interesting that PBL of some
donors showed a much higher binding to DC-SIGN-His-coated beads. DC-SIGN-specific binding was calculated by subtracting binding data determined in the presence of mannan. Determination of specific binding with EGTA and control-His beads was comparable with binding in the presence of mannan (Fig. 5B). PBL isolated from a donor whose PBL have a high DC-SIGN-binding capacity (22%) were labeled with subset-specific mAb to characterize the cells that bind DC-SIGN (Fig. 5D). All cell subsets displayed a distinct percentage of cells that were positive for DC-SIGN binding, which was highest for B cells (Table 1). Of the B cells present in the PBL preparation, ~25% are able to bind DC-SIGN beads. All the different subsets contribute to the overall binding percentage of 20.7%. Similar results were obtained when positive and negative FACS-sorted T cells, B cells, and NK cells were analyzed for DC-SIGN binding (data not shown).

To determine whether ICAM-3 plays a role in the binding of soluble DC-SIGN-His-coated beads to PBL, binding studies were performed in the presence of ICAM-3-blocking antibodies or by preblocking the DC-SIGN-coated beads with soluble ICAM-3. Although blocking ICAM-3 hardly affected the percentage of PBL that bound DC-SIGN-His-coated beads (data not shown), it reduced the MFI of binding PBL by 35%. In addition, preblocking the DC-SIGN-His-coated beads with soluble ICAM-3 resulted in a 34% reduction in the MFI of the binding PBL (Fig. 5D). As only a relatively low percentage of PBL bound DC-SIGN-coated beads, and the percentage of binding T cells in the PBL population was even lower (Table 1), the T cell line HSB-2 was used as a model to determine whether ICAM-3 on the T cell interacts with the DC-SIGN-coated beads. On average, ~80% of HSB-2 cells bound DC-SIGN-His-coated beads (data not shown). Preblocking DC-SIGN-His-coated beads with soluble ICAM-3, as blocking ICAM-3 on the cell surface with antibodies, significantly reduced binding of the DC-SIGN-His beads to HSB-2 cells (Fig. 5E).

Together, these data show that ~5% of PBL bind DC-SIGN beads and that binding is at least partly mediated by ICAM-3. The cells that bind DC-SIGN beads comprise all major cell subsets of PBL.
DC-SIGN-binding capacity parallels blocking capacity

As the number of PBL that bind DC-SIGN beads differs between donors, we tested whether this difference in binding could explain why some MLR are blocked by mAb against DC-SIGN, and others are not. PBL obtained from the donor characterized in Table 1/Figure 5C and PBL from another donor showing lower DC-SIGN binding were cocultured with the same DC isolated from five different allogeneic donors. Based on the definitions given above and in the legend of Figure 3B, all the MLR belong to the low-responder category.

Upon stimulation with immature DC, PBL with high DC-SIGN-binding capacity showed an overall decrease in T cell proliferation in the presence of blocking DC-SIGN and LFA-1 mAb (Fig. 6A). It has to be noted that addition of control mouse IgG itself influenced T cell proliferation. In contrast to the PBL showing a high DC-SIGN-binding capacity, DC-SIGN
NK cells (CD3–CD56–CD45RA–CD45RO+CD3+)

NKT cells (CD3+CD56+) with low DC-SIGN-binding capacity. The latter interaction is DC-SIGN-independent and therefore, not inhibitable by DC-SIGN mAb. The height of a MLR response is directly proportional to the number of T cells that are activated by APC. The number of T cells that are activated is, besides efficient costimulation, dependent on the integrated outputs of all TCR signaling events per cell. Consequently, a weak MLR is observed when fewer cells are able to evoke sufficient TCR signaling events compared with a strong MLR. Sufficient TCR signaling is attained by clustering the TCR-MHC complexes, as the affinity of the TCR is low [26]. Sustained signaling was also observed when the formation of the immunological synapse is inhibited [27]. DC-SIGN-ICAM-3 interactions prolong initial cell-cell contact and thereby prolong TCR signaling. Such prolonged cell-cell contact will result in a higher integrated signaling and thereby lowering the threshold for an immune response. Consequently, when more TCR-MHC are involved in the interaction, sufficient microclusters will be formed to initiate effective signaling and thereby minimizing the role for DC-SIGN-ICAM-3 interactions.

In conclusion, PBL isolated from the donor with high DC-SIGN-binding capacity did not block proliferation of PBL with low DC-SIGN-binding capacity. Nevertheless, LFA-1 mAb blocked T cell proliferation considerably.

In MLR driven by mature DC, the presence of DC-SIGN mAb also resulted in a decrease in overall T cell proliferation when PBL with high DC-SIGN-binding capacity were used (Fig. 5B). It is striking that PBL with low DC-SIGN-binding capacity displayed slightly enhanced T cell proliferation in the presence of DC-SIGN-binding mAb. The blocking effect of LFA-1 mAb on T cell proliferation is more pronounced in PBL with high DC-SIGN-binding capacity as compared with PBL with low DC-SIGN-binding capacity.

In conclusion, PBL isolated from the donor with high DC-SIGN-binding capacity were more prone to blocking by DC-SIGN mAb in MLR than PBL from the donor with lower DC-SIGN-binding capacity.

**DISCUSSION**

In the present study, we analyzed the relevance of DC-SIGN in DC-induced T cell proliferation. Our data indicate that its relevance in allogeneic T cell proliferation depends on the strength of the T cell response and on the percentage of PBL that are able to bind recombinant DC-SIGN.

A dependence on the strength of T cell response suggests an initiating role for DC-SIGN in DC-T cell interaction followed by other adhesive and costimulation events, which will boost T cell proliferation. The latter interaction is DC-SIGN-independent and therefore, not inhibitable by DC-SIGN mAb. The height of a MLR response is directly proportional to the number of T cells that are activated by APC. The number of T cells that are activated is, besides efficient costimulation, dependent on the integrated outputs of all TCR signaling events per cell. Consequently, a weak MLR is observed when fewer cells are able to evoke sufficient TCR signaling events compared with a strong MLR. Sufficient TCR signaling is attained by clustering the TCR-MHC complexes, as the affinity of the TCR is low [26]. Sustained signaling was also observed when the formation of the immunological synapse is inhibited [27]. DC-SIGN-ICAM-3 interactions prolong initial cell-cell contact and thereby prolong TCR signaling. Such prolonged cell-cell contact will result in a higher integrated signaling and thereby lowering the threshold for an immune response. Consequently, when more TCR-MHC are involved in the interaction, sufficient microclusters will be formed to initiate effective signaling and thereby minimizing the role for DC-SIGN-ICAM-3 interactions.

The proliferation data show that the degree of inhibition of T cell proliferation by LFA-1 and DC-SIGN-blocking mAb depends on the DC maturation state. Moreover, when using immature DC, DC-SIGN-blocking mAb only have an effect on some of the MLR that are categorized as weak MLR (<60% of PBL proliferate). Therefore, the variable blocking potential of DC-SIGN mAb on the MLR, as shown in this study and by Granelli-Piperno et al. [16], is explained by testing strong MLR.

**TABLE 1. Characterization of DC-SIGN-His-Binding PBL by Double-Triple Labeling FACS Analysis**

<table>
<thead>
<tr>
<th>Cell population</th>
<th>% Binding</th>
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PBL from the same donor as described in Figure 5B were incubated with DC-SIGN-His-coated beads for 30 min at 37°C. Subsequently, the cells were labeled with FITC- and/or PE-conjugated mAb for 20 min at RT where indicated. Samples were analyzed by flow cytometry. Data are percentages of cells within the indicated cell populations that displayed specific binding to the DC-SIGN-His-coated beads.

mAb did not block proliferation of PBL with low DC-SIGN-binding capacity. Nevertheless, LFA-1 mAb blocked T cell proliferation considerably.

In MLR driven by mature DC, the presence of DC-SIGN mAb also resulted in a decrease in overall T cell proliferation when PBL with high DC-SIGN-binding capacity were used (Fig. 5B). It is striking that PBL with low DC-SIGN-binding capacity displayed slightly enhanced T cell proliferation in the presence of DC-SIGN-binding mAb. The blocking effect of LFA-1 mAb on T cell proliferation is more pronounced in PBL with high DC-SIGN-binding capacity as compared with PBL with low DC-SIGN-binding capacity.

In conclusion, PBL isolated from the donor with high DC-SIGN-binding capacity were more prone to blocking by DC-SIGN mAb in MLR than PBL from the donor with lower DC-SIGN-binding capacity.

**Fig. 5.** Characterization of DC-SIGN-His binding to PBL. (A) The binding of PBL to DC-SIGN-His construct, in free form or coated to streptavidin-coated fluorescent beads, was determined. PBL were allowed to bind a 10-μg/ml recombinant soluble DC-SIGN-His construct for 30 min at 37°C, followed by incubation with Penta His Alexa-488 for 30 min at 37°C. For the beads assay, PBL were incubated for 30 min at 37°C with DC-SIGN-His-coated beads and subsequently analyzed by flow cytometry. Aspecific binding was determined by pretreatment of the DC-SIGN-His construct with 100 μg/ml mannan. Data are percentages of PBL isolated from several donors that displayed specific binding to the DC-SIGN-His construct. (B) Determination of aspecific binding of DC-SIGN-His beads to PBL by incubation of PBL with control-His beads or preincubation of DC-SIGN-His beads with 100 μg/ml mannan or 5 mM EGTA. One typical result out of 10 is depicted. (C) Characterization of DC-SIGN-His binding PBL by double/triple labeling FACS analysis. PBL isolated from a donor whose PBL showed high DC-SIGN-binding capacity were incubated with DC-SIGN-His-coated beads for 30 min at 37°C. Subsequently, the cells were labeled with FITC- and/or PE-conjugated mAb for 20 min at RT where indicated. Samples were analyzed by flow cytometry, and FACS pictures are depicted. Left column represents FACS pictures with the specific cell populations gated (Gate A, B, or C with percentage positive cells) for testing binding capacity to DC-SIGN-His beads. Right columns represent binding results of the specific cell populations to DC-SIGN-His beads (percentage binding indicated in picture). SSC, Side-scatter; FSC, forward-scatter. (D) DC-SIGN-His beads were preincubated in medium or in medium supplemented with 100 μg/ml soluble ICAM-3. The DC-SIGN-His beads preincubated in medium were allowed to bind to PBL, which were preincubated in medium or in medium supplemented with 20 μg/ml ICAM-3-blocking antibodies CBR3/1 and -3/2 (anti-ICAM-3 Ab). DC-SIGN-His beads preincubated in medium supplemented with soluble ICAM-3 were added to PBL preincubated in medium (soluble ICAM-3). Aspecific binding was determined by incubating PBL with DC-SIGN-His beads in the presence of 5 mM EGTA. PBL were allowed to bind DC-SIGN-His-coated beads for 30 min at 37°C. The mean cell fluorescence of DC-SIGN-His beads was determined by FACS analysis, and the percentages of the medium control value were calculated. Data are mean ± SD of three experiments. Significant difference from medium control, as determined by ANOVA and Dunnett’s test: *, P < 0.05. (E) The experiments shown in D were repeated using the T cell line HSB-2 instead of PBL. The mean cell fluorescence was determined by FACS analysis, and the percentages of the medium control value were calculated. Data are mean ± SD of three experiments. Significant difference from medium control, as determined by ANOVA and Dunnett’s test: **, P < 0.01.
or using mAb that do not bind to the carbohydrate-recognition domain of DC-SIGN.

It is interesting that the degree of inhibition of T cell proliferation by LFA-1 mAb is also dependent on the strength of the T cell response. In MLR driven by mature DC, LFA-1 mAb could only block MLR with a weak response.

The kinetic-blocking experiments indicate that DC-SIGN and LFA-1 exert initial effects in DC-T cell communication, which are dominated readily by other adhesive and costimulatory mechanisms. Such a dominating effect will be more pronounced with mature DC in comparison with immature DC because of their stronger T cell stimulation capacity [28, 29]. Indeed, relatively more low-responder MLR are observed when immature DC are used as T cell stimulators compared with mature DC as T cell stimulators (Fig. 3B). In contrast to DC-SIGN and LFA-1 mAb, T cell proliferation could be blocked over a long period of time by blocking CD6 mAb, which indicates CD6 plays an important and long-lasting role in DC-T cell contacts.

An initial effect of LFA-1 in DC-T cell communication is supported by a study of Bachmann et al. [30]. They demonstrate that LFA-1 induces T cell activation by promoting adhesion of T cells to APC instead of providing long-lived costimulatory signals [30]. Another study also indicates that LFA-1 is not able to provide strong costimulatory signals such as CD28, which is the most dominant costimulatory molecule [31].

The dominating role of other adhesive and costimulatory mechanisms may play a minor role when less professional APC are used such as THP-1 cells differentiated into DC-SIGN+ cells. These DC-like cells induce T cell proliferation, which is blocked by DC-SIGN mAb [13]. Probably the effect of DC-SIGN on T cell proliferation is more easily over-ruled than that of LFA-1 because of the transient nature of the DC-SIGN/ICAM-3 interaction. It has been reported that DC-SIGN/ICAM-3-dependent adhesion between DC and T cells varies and reaches an optimum after 20 min [7]. In addition, DC-SIGN can recruit LFA-1 to the contact site and shift from initial transient DC-SIGN-ICAM-3 interactions to more stable LFA-1-ICAM-3 interactions [25]. Furthermore, ICAM-3 is a dynamic molecule, as it is recruited rapidly to the APC-T cell contact site followed by a redistribution to the outer zone of the cell-cell interface upon contact stabilization [4].

In agreement with previous studies [32, 33], our DC-T cell conjugates do not show an enriched distribution of DC-SIGN in the DC-T cell contact area, regardless of the use of immature or mature DC and of the distribution pattern of ICAM-3. Apparently, sufficient DC-SIGN molecules are present on the DC membrane to establish a transient interaction with ICAM-3.

Funatsu et al. [34] reported that ICAM-3 molecules isolated from human PBL only contain 6% high mannose-type oligosaccharides of the total ICAM-3 oligosaccharide pool. The authors of this study suggest that these high mannose-type oligosaccharides are expressed on a special subset of T cells [34]. However, we could not pinpoint the DC-SIGN-binding cells to one specific T cell population. Instead, we observed that all cell populations analyzed bind DC-SIGN to a similar
degree, except for B cells, which showed a much higher specific binding (Table 1). Thus, besides major T cell subsets, DC-SIGN beads also bind B cells and NK cells. Interactions between DC and B cells and DC and NK cells modulate DC function and can indirectly have an impact on T cell response [35, 36].

It seems likely that high mannose-type oligosaccharides are expressed at low levels on all T cells and underlie the transient binding of DC-SIGN to T cells. This concept is supported by the finding that DC-SIGN-His-coated beads were superior in binding to PBL as compared with soluble DC-SIGN-His. Multiple DC-SIGN-His molecules are attached to each fluorescent bead, endowing it with a high avidity, thus improving stable interactions between the bead and the low number of specifically glycosylated ICAM-3 molecules expressed on PBL. DC-SIGN-His, in soluble form, may at best form tetramers [37] and thus, will have a lower avidity.

PBL isolated from some donors showed a considerable higher binding to DC-SIGN-His-coated beads than other donors. This high-binding potential was consistent over time, as PBL with a high DC-SIGN-binding capacity from a specific donor still showed a high specific binding to DC-SIGN-His coated beads when measured 6 and 18 months later (data not shown).

Snyder et al. [38] found submicromolar binding affinities when using ICAM-3, which was produced in the mouse cell line NSO. Glycosylation is important for reactivity of DC-SIGN, as Snyder et al. [38] show in their paper for gp120, the other ligand of DC-SIGN. Mouse cells are known to glycosylate incompletely in vitro-produced proteins. As DC-SIGN recognizes high mannose-type oligosaccharides, it is not surprising that those authors find submicromolar-binding affinities. The ligand is simply not expressed at sufficient levels. As reported above, ICAM-3 isolated from human PBL is expressing only 6% high mannose-type oligosaccharides [34]. Moreover, the same authors show that for binding, tetrameric DC-SIGN is adamantly [38]. Chimeric Fc-ICAM-3 is believed to be dimeric, which adds to their poor binding results. Geijtenbeek et al. [7] used beads that harbor multiple Fc-ICAM-3 ligands for binding studies to circumvent those valency issues. It seems likely that the donors whose PBL bind DC-SIGN with high capacity express an increased level of specifically glycosylated ICAM-3 and possibly other specifically glycosylated ligands. Changes in glycosylation can be related to the differentiation status of a cell, as a T cell displays several glycosylation patterns during its development [39]. Ryan et al. [19] showed a preferential binding of naïve CD45RA+ cells to DC-SIGN-His. However, in our study no clear differences were observed between naïve/resting cells (CD45RA+, CD62L+) and memory/activated cells (CD45RO+, CD25+) [40–42].

PBL, with a high DC-SIGN-binding capacity, were more prone to DC-SIGN-blocking mAb in the MLR, even when mature DC were used to stimulate the T cells. In these MLR, DC-SIGN can bind firmly to a higher percentage of T cells and may have a prolonged effect on these cells compared with cells that bind transiently. In a MLR, only 1–10% of the T cells are able to respond to the allogeneic MHC-peptide complexes expressed on DC. Therefore, only a relatively small number of T cells binding firmly to DC-SIGN might be required to have a substantial effect in the MLR. Because of the higher frequency of firm binding cells in the donor whose PBL have a high DC-SIGN-binding capacity, the effect of blocking DC-SIGN in the MLR is observed more rapidly. Accordingly, the level of DC-SIGN-binding capacity of PBL may explain why not all weak MLR stimulated by immature DC are down-regulated by DC-SIGN-blocking mAb.

The MLR experiments in Figure 6 show that the inhibitory action of DC-SIGN mAb is not DC donor-specific or dependent on the level of DC-SIGN expression. This can be concluded from the fact that the use of the same DC donor and therefore, the same DC-SIGN expression level, in combination with two PBL donors, resulted in different effects of the DC-SIGN mAb.

Our data indicate that DC-SIGN is involved in early contacts between DC and T cells. It is tempting to speculate that DC-SIGN is involved in the initial exploratory contact of T cells with DC in a similar way as described for ICAM-3 [4]. In this regard, DC-SIGN may act like a selectin by mediating the “rolling” of T cells over DC in a similar way as selectins do by mediating the rolling of T cells along the endothelium [43]. Future dynamic studies performed in a three-dimensional setting could address this phenomenon.

Summarizing, this study indicates an initial and transient role for DC-SIGN in T cell proliferation, which becomes apparent in weak MLR and when the percentage of DC-SIGN-binding PBL is high.

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