INVESTIGATIONS INTO MECHANISM OF ACTION OF CD24-BASED THERAPY AND SELECTIVE TARGETING FOR MCL

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Abstract

Recently, CD24 has been proposed as a novel therapeutic target for B-cell leukemias and lymphomas, with our own work highlighting potent macrophage-mediated phagocytosis of Mantle Cell lymphoma (MCL) cells upon CD24 antibody treatment in vitro. However, our earlier work led us to question the validity of the proposed role of CD24/Siglec-10 as an immune checkpoint (IC) that regulates phagocytosis. In this study we aimed to address this question on IC activity of CD24/Siglec-10 and at the same time develop a potential cancer cell-selective CD24-based therapy for MCL. In brief, we generated human bispecific single-chain variable fragments (bi-scFv) designed to bind simultaneously to CD24 and CD19 (CD24xCD19 and CD19xCD24). These constructs lack an Fc-domain and were intended to have high avidity and restricted IC inhibitor activity towards dual-antigen expressing cancer cells, in the absence of any FcR-mediated activity, with minimal binding and activity to single CD19 or CD24-positive cells. Thus, these constructs should effectively block CD24 only on MCL cells, allowing evaluation of potential IC activity. In line with this proposed mode-of-action, CD24xCD19, but not CD19xCD24, selectively bound only to double-positive CD24⁺CD19⁺ MCL cell lines. The binding of CD24xCD19 was blocked by pre-incubation with CD24 and/or CD19 mAb. Treatment of MCL cell lines with CD24xCD19 yielded a significant increase in phagocytosis, yet 90% reduced compared to treatment with CD24 mAb clone SN3, containing a murine IgG1. Thus, CD24 targeting may contain a minor ICI component. However, upon partial SIGLEC10 knock down in CD34⁺-derived macrophages, no correlation between Siglec-10 expression and phagocytosis levels was found. Further, recombinant human Siglec-10 protein lacked binding to some CD24⁺ cell lines, in which CD24 mAb treatment induced high levels of phagocytosis. Taken together, the phagocytic effect observed upon CD24 antibody treatment is primarily Fc-mediated and, despite there being some remaining IC activity when using CD24xCD19, this residual activity seems to be Siglec-10 independent.
Introduction

In the context of hematological malignancies, CD24 expression has been reported in most subtypes of leukemia and lymphoma (1,2). In general, high expression of CD24 associates with poor prognosis (1). In line with this, we previously reported that CD24 expression negatively correlated with survival in several non-Hodgkin's B-cell lymphomas (B-NHLs), including follicular lymphoma and mantle cell lymphoma (MCL) (chapter 2). A recent study also described that CD24-high diffuse large B cell lymphoma (DLBCL) patients had worse progression-free and overall survival than the CD24-low group (3).

Since CD24 was described as a novel “don’t eat me signal” with relevance in carcinoma (4), more groups have investigated the possibility of targeting CD24 for the treatment of lymphomas and leukemias (2,3). In older studies, treatment of EBV-transformed B cells with unconjugated CD24 antibodies (IOB3-IgG1) also improved survival of immunodeficient mice approximately by 40% at 30-42 days post tumor injection (5). We previously described that CD24 antibody (mAb) treatment (clone SN3 IgG1) triggered high levels of phagocytosis, with a superior effect than CD47 InhibRx mAb, in both MCL cell lines and primary MCL blasts (chapter 2). Recently, in May 2023, the FDA has approved a phase I dose-finding study of ATG-031, an anti-CD24 IgG1-based antibody, in patients with advanced solid tumors or B-NHL (6). Overall, these studies suggest a potential role of CD24-based therapy for the treatment of certain B-cell malignancies, with special relevance for those lacking response to current therapeutic options, such as MCL.

Similar to CD20 targeting (e.g., with Rituximab), the anti-CD24 mAb can bind directly and cluster CD24 to induce apoptosis or recruit NK cells or phagocytes via their Fc receptors to induce ADCC or ADCP (7). Further, CD24 antibody treatment might be used to disrupt CD24-Siglec-10 interaction to block CD24 checkpoint activity and reactivate the phagocytic activity of tumor-associated macrophages (4). With all these possible mechanisms of action (MoAs) highlighting the versatility of CD24-based therapy, the detailed mechanism behind CD24 mAb treatment to achieved tumor growth control in lymphoma has not been completely elucidated. For instance, the use of anti-CD24 mAb (clone ML5) in our previous study yielded Fc-dependent phagocytosis (ADCP), although clearly disrupting CD24/Siglec-10 interaction in vitro (chapter 2). Further, we and others have failed to demonstrate that Siglec-10 blockade is crucial to disrupt CD24-Siglec-10 interaction in MCL. Therefore, although all published data so far show that targeting CD24 via mAb in several cancer types reduces tumor cell growth, more efforts are needed to elucidate the underlying mechanism(s) behind this effect.

Notably, although CD24 can clearly be considered a promising target for B-cell leukemias and lymphomas, the main challenge of CD24 mAb treatment is the expected toxicities due to the high expression of CD24 in virtually all human tissues and other cells from the immune system (1). In this respect, CD24 mAb off-target killing of DCs in secondary lymphoid organs is likely to induce an important expansion of T cells with the death of the host (8). One possible approach to reduce the likely wide-spread off-tumor effects of CD24-
based therapy is the tumor-specific targeting of CD24 blockade by using bispecific antibodies (bsAbs), as already described for CD47-based therapy (9,10). By combining CD24 with a tumor-targeting antigen (e.g., CD20 or CD19 in B cell blasts), selective targeting of CD24 might be achieved enhancing both the efficacy and safety of CD24-based therapies.

In this study, we developed human bi-specific single-chain variable fragments (bi-scFv) antibody formats targeting both CD24 and CD19. This allows for the selective targeting of CD24-based immunotherapy specifically to MCL blasts (CD19+) and reduces off-tumor effects. Importantly, as the bi-scFv lacks an Fc-domain any functional phagocytic effect should be attributed to ICI activity. This, together with further in vitro functional assays including the SIGLEC10 KO in monocyte-derived macrophages, allowed us to shed more light about the dominant MoA behind CD24-targeted therapy in MCL.
Results and discussion

CD24xCD19, but not CD19xCD24, binds to CD24+CD19+ double positive cell lines, with a higher affinity for the CD19 epitope and disrupts CD24/Siglec-10 interaction

To test whether the CD24xCD19 and CD19xCD24 constructs (Figure 1A) could bind simultaneously to both targets, a panel of different cell lines with variable expression of both CD24 and CD19 was used (Figure 1B). Specifically, cells lacking expression (CEMEV), cells ectopically expressing CD19 (CEMCD19), cells expressing only CD24 (SK-BR-3), and cells expressing CD24^{high}CD19^{+} (UPN-1, HBL-2) or CD24^{low}CD19^{+} (SU-DHL-4, SU-DHL-6, U-2932) were included. Interestingly, when incubated with HBL-2 (CD24^{high}CD19^{+}) only CD24xCD19 but not CD19xCD24 construct proved to bind to these cells, as measured by detecting the N-terminal HA affinity tag (Figure 1C). Thus, we selected CD24xCD19 construct for further experiments. In line with the MoA of the bsAb construct, the binding of CD24xCD19 was higher when both epitopes (CD24 and CD19) were present, such as in the case of MCL cell lines UPN-1 and HBL-2 (purple) (Figure 1D). In that case, binding of CD24xCD19 to the cells could be detected from 1 µg/mL of construct, although with small differences between the MCL cell lines (Figure 1E). Also, a smaller binding was detected towards the CD19^{+}CD24^{low} group of cell lines (orange), (Figure 1D). Of note, the fact that CD24xCD19 binds to CD19 only (CEM^{CD19}), but not CD24 only (SK-BR-3) cell lines suggests that the binding of CD24xCD19 is primarily driven by the CD19 scFv, but still enhanced by simultaneous CD19 and CD24 expression.

Nevertheless, binding of CD24xCD19 specifically to both CD24 and CD19 epitopes was confirmed by the reduction in binding upon pre-incubation of HBL-2 (Figure 1F,G) and UPN-1 (Figure 1G) with CD24 mAb or CD19 mAb. In support of the higher binding affinity for CD19, pre-incubation with CD19 mAb (red) completely abrogated binding of CD24xCD19 to both HBL-2 and UPN-1 (Figure 1G). Pre-incubation with CD24 mAb (clone SN3) (blue) only partially inhibited binding of CD24xCD19. Finally, as the precise epitope of CD24xCD19 on CD24 is not defined, the ability of CD24xCD19 to block CD24/Siglec-10 interaction was tested by its ability to inhibit binding of rhSiglec-10 (His tagged) protein to HBL-2. Pre-incubation with CD24xCD19 partially prevented the binding of rh Siglec-10 protein to HBL-2 (Figure 1H, I) similar to CD24 mAb clone SN3 (chapter 2).

Taken together, CD24xCD19 but not CD19xCD24 binds CD24^{+}CD19^{+} double positive cell lines. The simultaneous expression of both CD19 and CD24 enhanced the binding ability of the construct, probably due to a higher avidity of dual binding. Further, CD24xCD19 was able to (partially) disrupt binding of rhSiglec-10 to HBL-2. Thus, CD24xCD19 can be used to target CD19^{+}/CD24^{+} cells and to check the role of CD24/Siglec-10 interaction in macrophage-mediated phagocytosis.
Figure 1. Characterization of CD24xCD19 binding. (A) Schematic representation of CD24xCD19 and CD19xCD24 constructs formed by the simultaneous fusion of CD24-blocking scFv and CD19-blocking scFv through a (GS4)3 linker (B) The relative surface expression levels indicated as MFI values of CD24 and CD19 within a panel of cell lines. Data was normalized to the MFI values of HBL-2. n=3, Mean+SD (C) Representing histograms for the binding of CD24xCD19 (blue) and CD19xCD24 (purple) to HBL-2 (left), measured as anti-HA binding. (D) Binding of 25 µg/mL of CD24xCD19 to a panel of cell lines, measured as anti-HA binding and represented as fold-change in MFI values compared to isotype. cells lacking expression (black), cells ectopically expressing CD19 (blue), cells expressing only CD24 (red), and cells expressing CD24highCD19+ (purple) or CD24lowCD19+ (orange) are included. n=3, Mean + SD (E) Binding capacity of CD24xCD19 to HBL-2 and UPN-1 cell lines (CD24highCD19+) representatively in a concentration-dependent way. (F) Representing histograms for the binding of 25 µg/mL of CD24xCD19 to HBL-2 cells upon pre-incubation with CD19 mAb (blue), CD24 (SN3) (yellow) mAb or both (combi, green), measured as anti-HA binding. (G) Quantification of CD24xCD19 to HBL-2 and UPN-1 upon pre-incubation with CD19 mAb (red), CD24 (SN3) (blue) mAb or both (combi, purple), measured as anti-HA binding. MFI values were normalized to anti-HA binding alone. n=3, Mean+SD (H) Representing histograms for the binding of rhSiglec-10 protein (His-tagged) to HBL-2 cells with (blue) and without (red) previous incubation with the CD24xCD19 construct. (I) Quantification of the binding of rhSiglec-10 protein (His-tagged) to HBL-2 (CD24high) cells with and without previous incubation with the CD24xCD19 construct. MFI values were normalized according to Siglec-10 binding alone. n=3, Mean+SD.
CD24xCD19 treatment induced a significant, but highly reduced, increase in phagocytosis of MCL cells compared to CD24 SN3 clone (murine IgG1) mAb

Pre-treatment of MCL cell lines with different concentrations of CD24xCD19 induced a modest yet significant increase of 4-10% in the phagocytic uptake of HBL-2 (Figure 2A) and UPN-1 (Figure 2B) by M2c macrophages. In UPN-1, combination of CD24xCD9 did not significantly increase the pro-phagocytic effect of treatment with 10 ng/mL of opsonizing CD20 antibody rituximab (RTX), whereas it did for two of the concentrations tested in HBL-2 (Figure 2A). Of note, CD24xCD19 was not purified and was used in supernatant form. However, no significant differences were observed between medium (no treatment) and medium supernatant (treatment with supernatant control) (Figure 2C). The latter refers to supernatant harvested under the same conditions as CD24xCD19. The fact that CD24xCD19 triggered a small but still significant increase in phagocytosis suggests that, in line with previous publications (2,4), CD24 can be targeted to increase the phagocytic uptake of CD24+ cells. Importantly, despite being significant, the effect induced by CD24xCD19 was significantly reduced compared to the more than 60% increase in phagocytosis induced by IgG-containing CD24 mAbs (both SN3 and ML5 clones) (chapter 2). In fact, when the phagocytic uptake induced by CD24xCD19 was directly compared to that obtained by CD24 mAb (SN3) clone (Figure 2D), more than a 90% reduction in effect was observed for the bi-scFv compared to the full mAb, for both UPN-1 and HBL-2 (Figure 2E). Therefore, the likely reason why CD24 mAb (SN3) has a superior capacity to induce phagocytosis than CD24xCD19 is the presence of an Fc-domain. Previously, we could not assign SN3 activity definitively to CD24 checkpoint or Fc-mediated mechanisms (chapter 2). However, according to these new data, the Fc-domain itself would have an important contribution to the effect of CD24 mAb treatment. If this is the case, CD24-based therapies might be further optimized by exploring Fc-engineering strategies, as well as IgG1-bsAb formats, in order to enhance the therapeutic effect of the antibody while minimizing off-tumor effects. In the near future, a phase I study using an anti-CD24 IgG1 antibody (ATG-031) will be initiated in the USA, also for B-NHL patients. The results from this study will shed more light on the safety and tolerability of using anti-CD24 IgG1 as monotherapy in the clinic.

Finally, the effect induced by CD24xCD19 seems to be specific of CD24 binding, as no significant increase in phagocytosis was detected when CD19 was targeted in HBL-2 with a CD19 mAb (Figure 2F). However, in line with the MoA of the construct, CD24xCD19 treatment requires not only CD24 but also CD19 expression to be effective, as treatment of CD24+ CD19+ SK-BR-3 cells did not trigger any effect (Figure 2G). Thus, despite its lower capacity to trigger phagocytosis compared to SN3, the effect induced by CD24xCD19 seems to be at least specific of CD24 targeting and selective towards CD24+CD19+ cell lines.
Figure 2. Phagocytic uptake of MCL cell lines induced by CD24xCD19. (A) Percentage increase in phagocytosis of HBL-2 cells by M2c macrophages triggered by different concentrations of CD24xCD19 alone (red) or in combination with 10 ng/mL of rituximab (RTX) (blue) compared to basal phagocytosis (gray). (B) Percentage increase in phagocytosis of UPN-1 cells by M2c macrophages triggered by different concentrations of CD24xCD19 bi-scFv alone (red) or in combination with 10 ng/mL of rituximab (RTX) (blue) compared to basal phagocytosis (gray). (C) Comparison in percentage of phagocytosis obtained for untreated cells (medium, gray) and cells treated with supernatant control lacking any construct (supernatant, blue). (D) Flow cytometry diagrams showing the percentage of cell trave violet (CTV) and CD11b positive cells within medium, CD24xCD19 (25 µg/mL) and CD24 mAb (SN3 clone) samples during a phagocytosis assay. (E) Comparison of delta increase in phagocytosis between CD24 mAb and CD24xCD19 (25 µg/mL) in both UPN-1 and HBL-2. (F) Percentage increase in M2c-phagocytosis of HBL-2 cells for untreated (medium, black), isotype (gray), CD24 mAb (blue) and CD19 mAb (red) treatment. (G) Percentage increase in phagocytosis of SKBR-3 cells for untreated (medium), CD24 mAb (blue) and CD24xCD19 (25 µg/mL, red) treatment. Where indicated, * = p < 0.05; ** = p < 0.01; *** = p < 0.001, ns= non-significant
The extent of phagocytosis of MCL cell lines did not correlate with Siglec-10 expression levels on TAMs

To evaluate the impact of Siglec-10 expression on the phagocytic uptake of MCL cell lines observed upon CD24 blockade, especially with CD24 mAb (clone SN3), Siglec-10 expression on M2c-type macrophages was modulated using several approaches. Firstly, we observed that addition of TGF-β to the polarization media increased the levels of Siglec-10 expression on the cell surface (Figure 3A), in line with previous data (Barkal et al). Interestingly, M2c macrophages with a Siglec-10-low phenotype (IL-10 only) showed increased phagocytic capacity than the ones Siglec-10-high phenotype (IL-10 + TGF-β) for all the samples, including CD24 mAb and CD24xCD19 treated ones (Figure 3B). This suggests a higher phagocytic capacity of these macrophages, but in contrast to what we expected, both CD24 mAb and CD24xCD19 treatment also yielded higher phagocytosis levels on these Siglec-10-low macrophages.

To exclude the possibility of additional phenotypic changes due to use of different polarization protocols, we next evaluated phagocytosis in CD34+ derived macrophages that were subjected to SIGLEC10 CRISPR/Cas9 knock-out (KO) protocol (Figure 3C). The KO efficiency of three individual experiments is shown in Figure 3D, with a partial > 80% knock-down of Siglec-10 surface expression. When the phagocytic capacity of these macrophages was evaluated towards MCL cell lines, no significant difference was observed in the phagocytic uptake of either HBL-2 (Figure 3E) nor UPN-1 (Figure 3F) between wild-type (WT) and Siglec-10 KO macrophages. Similar results were obtained when comparing the delta increase in phagocytosis achieved by CD24 (SN3 clone) and CD24xCD19 treatment between WT and SIGLEC10 KO macrophages (Supplementary material Figure 2). Indeed, Siglec-10 surface expression on M2c-derived macrophages did not significantly correlate with phagocytic uptake of HBL-2 (Figure 3G) and UPN-1 (Figure 3H) upon CD24 mAb treatment. Interestingly, in the case of UPN-1 a slightly positive tendency was detected between reduced Siglec-10 expression and higher phagocytic uptake. Notably, this finding contradicts with the low-level binding of recombinant human Siglec-10 protein (rhSiglec-10) to UPN-1 compared to HBL-2 (Figure 3I). Overall, it seems that HBL-2 and UPN-1 cells, despite being both MCL cell lines might present differences in their glycosylation pattern, therefore affecting the interaction between CD24 and Siglec-10. However, this is just a hypothesis as glycosylation state of these cell lines hasn’t been evaluated here. Although rhSiglec-10 protein binding cannot be directly compared to the binding of endogenous Siglec-10 expressed by macrophages, HBL-2 interacts strongly with recombinant Siglec-10 and yet, the phagocytic effect of CD24 mAb treatment did not correlate to Siglec-10.

Interestingly, our data suggest that CD24 may be a checkpoint in innate immunity, but disruption of CD24/Siglec-10 interaction seems not a relevant MoA behind the phagocytic uptake observed in our experiments. The interaction of CD24 with Siglec-10 is widely described in the literature (11), but it has not been always the proposed mechanism.
behind the anti-tumor effects observed upon CD24 mAb treatment (12). In this regard, inhibition of STAT3 signaling (13,14), inhibition of the Ras pathway (15), or the modification of intertumoral cytokine microenvironment (16) have been also described. As deeply discussed in chapter 6, the hypothesis of CD24/Siglec-10 interaction as don’t eat me signal is actually marginally supported in literature highlighting the need of further pre-clinical studies.

Figure 3. Exploring the role of Siglec-10 in CD24 mAb mediated phagocytosis. (A) Surface Siglec-10 expression plotted as MFI values on M2c macrophages polarized with 50 ng/mL of IL-10 only or in combination with TGF-β. (B) Percentage of M2c-mediated phagocytosis (same donor as in (A)) upon 2h co-culture with HBL-2 cells (medium) and HBL-2 treated with 1 µg/mL CD24 mAb (αCD24), 5 µg/mL CD24xCD19 bi-scFv or 10 ng/mL rituximab (RTX). Phagocytosis performed by M2c IL-10 only (gray) and IL-10 + TGF-β (blue) is shown. (C) Representative histograms for Siglec-10 expression on WT and SIGLEC10 KO (guide AB) on M2c CD34-derived macrophages. (D) Siglec-10 expression plotted as MFI values on WT and SIGLEC10 KO (guide AB) M2c CD34-derived macrophages. (E) Percentage of M2c CD34-derived macrophage phagocytosis (WT and SIGLEC10 KO) after 2h co-culture with HBL-2 cells. (F) Percentage of M2c CD34-derived macrophage phagocytosis (WT and SIGLEC10 KO) after 2h co-culture with UPN-1 cells. (G) Correlation between Siglec-10 expression on M2c macrophages and increase of phagocytosis (shown as delta phagocytosis compared to medium control) upon CD24mAb treatment (1 µg/mL) of HBL-2 cells. Siglec-10 expression was normalized to the highest value. Pearson’s r = 0.0822 and linear regression p > 0.05. (H) Correlation between Siglec-10 expression on M2c macrophages and increase of phagocytosis (shown as delta phagocytosis compared to medium control) upon CD24mAb treatment (1 µg/mL) of UPN-1 cells. Siglec-10 expression was normalized to the highest value. Pearson’s r = 0.3683 and linear regression p > 0.05. (I) Binding of increasing concentration of rhSiglec-10 protein (His tagged), detected as anti-His antibody binding (MFI) to UPN-1 (black) and HBL-2 (red) MCL cell lines. n=3, Mean + SD
**Conclusion**

This study supports the potential of therapeutic targeting of CD24 for the treatment of hematological malignancies, in particular MCL. Future studies will be performed to fully characterize and evaluate the advantages of using bi-scFv formats in increasing on-tumor activity and reducing off-tumor effects of CD24 targeting. Throughout this work we have validated a CD19-selective re-targeting of CD24-based therapy which could be further expanded towards an IgG1-bsAb. The inclusion of an Fc-domain might be of great relevance as, according to our data, CD24 mAb (SN3) seems to trigger phagocytosis primarily through ADCP rather than checkpoint activity. In agreement with the latter, we did not observe any significant correlation between Siglec-10 expression on macrophages and CD24-targeted induced phagocytosis supporting the hypothesis that the phagocytic effect observed upon CD24 antibody and/or CD24xCD19 mediated treatment might be due to some checkpoint activity that is likely Siglec-10 independent.

**Materials and Methods**

**Reagents**

Anti-CD24 (Clone SN3) was purchased from Novus Biologicals (LLC, Littleton, CO, USA). Anti-CD24 (Clone ML5), mouse anti-CD19 (Clone HIB19), anti-Siglec-10 mAb (Clone 5G6), anti-CD24-APC (Clone SN3), CD11b-Alexa Fluor 594 (M1/70), anti-CD19-BV785, anti-Siglec-10-APC, , anti-HA.11 epitope tag APC (Clone 16B12 were purchased from Biolegend (San Diego, CA, USA). Rituximab (RTX) was obtained from the Hematology Department of the University Medical Centre of Groningen (UMCG) (Groningen, The Netherlands). Penta-His-Alexa Fluor 647 antibody was obtained from Qiagen (Hilden, Germany). Recombinant human Siglec-10 protein (His-tagged) was obtained from Abcam (Cambridge, MA, USA). CellTrace™ CFSE and CellTrace™ Violet were obtained from Thermo Fisher Scientific (Cleveland, OH, USA). Anti-CD11b-APC (clone MEM-174), CD11b-FITC, granulocyte-macrophage colony-stimulating factor (GM-CSF), macrophage-colony-stimulating factor (M-CSF), interferon-γ (IFN-γ), and interleukin 10 (IL-10) were purchased from Immunotools (Germany). Lipopolysaccharide (LPS) was purchased from Sigma-Aldrich (Merck, MO, USA) and TGF-β was from Peprotech (Thermo Scientific, London, UK). Recombinant human Siglec-10 protein (His-tagged) was purchased from Abcam (Cambridge, MA, USA). TracRNA and HiFi Cas-9 Nuclease were obtained from IDT technologies (Coralville, Iowa, USA).

**Cell lines and culture conditions**

MCL cell lines UPN-1, HBL-2 and breast carcinoma cell line SK-BR-3 were obtained from the American Type Culture Collection (Manassas, VA, USA). HEK 293T cells and DLBCL cell lines U-2932, SU-DHL-4 and SU-DHL-6 were obtained from Deutsche Sammlung from Microorganism and Zellkulturen, (Braunschweig, Germany) and ATCC. All cells were cultured...
according to the supplier’s recommendation either in RPMI or DMEM (Lonza, Bio Whittaker BE12–604F and BE12–155F) supplemented with 10% fetal bovine serum (FBS, Gibco™ Fetal Bovine Serum, USA) at 37 °C in a humidified 5% CO2 atmosphere and were regularly tested for mycoplasma infections by PCR.

**Generation of bi-scFv constructs**

CD24-blocking tandem bi-scFv CD24xCD19 (Figure 1A, supplementary figure A) comprises an N-terminal Hemagglutinin tag, followed by a CD24-directed scFv antibody fragment (scFv CD24) derived from CD24mAb, a (G4S)3 linker and an in-frame CD19-targeting scFv (clone HIB19) in plasmid pRRL-SFFV. CD19xCD24 was similarly generated but contains the CD24scFv fragment in position 1 and the CD19scFv in position 2. The bi-scFv stable producer cell lines were generated by lentivirally-transducing HEK293 T cells with lentivirus encoding the corresponding bi-scFv construct. Lentivirus was generated by transfection of transfer plasmid pRRL-SFFV bi-scFv_GFP and helper plasmids in HEK293 cells using Fugene® HD reagent according to manufacturer’s instructions (Promega). After transduction and clonal selection, producer cells were cultured in X-CELL® 293 Serum-Free Medium (14571C-500ML, MERCK) and supernatant was collected at several time points from day 1 to day 8, filtered and stored at 4°C. The structure of the constructs was validated by western blot, being detected with an anti-HA primary antibody and secondary HRP-conjugated antibody (Supplementary Figure 1B). Further, non-reducing SDS-PAGE was performed to evaluate the degradation levels of the constructs (Supplementary Figure 1C). Concentration was determined using dot blot for HA-tag, with a dose-range of an HA-tag mAb of known concentration as calibration (Supplementary Figure 1D).

**Isolation of immune and CD34+ cells**

Monocyte-derived macrophages were obtained from whole blood (WB), whereas CD34+ cells were isolated from cord blood. In both cases, peripheral blood mononuclear cells (PBMCs) were isolated by density gradient centrifugation with Lymphoprep™ according to the manufacturer’s recommendations (STEMCELL Technologies, Vancouver, BC, Canada). For macrophage isolation, PBMCs were seeded in a 6-well plate at a density of 2.5x 10^6 cells/mL in RPMI 10% FBS containing the M0 differentiation cytokines GM-CSF or M-CSF (50 ng/mL each) for 7 days. To generate M1-type macrophages, M0 cells were treated with LPS (100 ng/mL) and IFN-γ (20 ng/mL) for 1 day. For M2c-type macrophages, M0 cells were primed with IL-10 (50 ng/mL) and TGF-β (50 ng/mL) for 2 days. Isolated CD34+ cells were counted and seeded in 6 or 12-well plates in Stemline II Hematopoietic Stem Cell Expansion medium (Sigma-Aldrich, #S0192) supplemented with SCF (100 ng/mL), FLT3 (50 ng/mL), IL-6 (10 ng/mL) and GM-CSF (30 ng/mL) and kept at a density of ~1 x 10^6 cells/mL. Cells were expanded for up to 14 days until total cell numbers reached >16 x 10^6 cells, and subsequently used for experiments.
INVESTIGATIONS INTO CD24/SIGLEC-10 AXIS

**Generation of SIGLEC KO in human derived M2c macrophages**

CRISPR/Cas9 KO of Siglec-10 in human donor derived CD34+ cells was performed using the Amaxa™ 4D-Nucleofactor Protocol for CD34+ cells (Lonza) and the P3 Primary Cell Nucleofection kit (LONZA V4XP-3024), program E0-100. Two crRNA molecules targeting the SIGLEC10 were purchased from IDT Technologies and complexed with tracRNA as previously described. The crRNA sequences used are indicated in Table 1. Post nucleofection, cells were seeded in 12-well plates in RPMI 10% FCS, 5% human serum and M-CSF (50 ng/ml), and incubated at 37°C, 5% CO2 achieve M0 differentiation. On day 9-10, M0 cells were polarized to M2c as previously described. Prior to phagocytosis assays, expression of M2c markers Siglec-10, and CD206 and CD163 (data not shown) were checked using flow-cytometry (CytoFLEX, Beckman Coulter, Fullerton, CA, USA) and CytExpert Software (Beckman Coulter). Phagocytosis was performed once SIGLEC10 knockout was confirmed.

**Table 1. crRNA sequences**

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**Target antigen binding by CD24-based bsAbs**

Binding of CD24xCD19 to a panel of CD24 and CD19 positive and negative cell lines was performed by incubating 100k cells with 25 µg/mL of bsAb supernatant for 30 min at 4 degrees. Cells were then washed and analyzed by flow cytometry (CytoFlex, BC). To analyze the activity profile of the generated bsAb formats, blocking activity of recombinant His-tagged human Siglec-10 (R&D systems) towards the binding of the bsAbs was evaluated on CD24 high HBL-2 and UPN-1. In brief, 50k cells were pre-treated with 2 µg/mL of Siglec-10 protein for 1h on ice. Next, 10 times molar ratio of bsAb supernatant was added and incubated for another 30 min at 4 degrees. Cells were finally washed x3 times with 2mL of PBS and analyzed by flow cytometry. Median fluorescence intensity was used to quantify the binding. Finally, bsAb binding was competitively inhibited using CD24 and CD19 mAbs by pre-treating CD24 and CD19 positive cell lines (HBL-2, UPN-1) with 1 µg/mL of CD24 SN3 and 1 µg/mL of CD19 (Clone HIB19) antibodies for 30 min at 4 degrees. Then, 25 µg/mL of bsAb supernatant was added and incubated for 30 min at 4 degrees. subsequently, cells were washed 3 times with 2mL of PBS and analyzed by flow cytometry. Median fluorescence intensity (MFI) was used to quantify the binding.

**Phagocytosis assay**

Flow cytometry-based phagocytosis assays were performed by co-culturing cancer cells and macrophages in RPMI 10% FCS at an Effector-to-Target (E:T) ratio of 1:3 (for MCL and DLBCL) or 1:1 (for carcinoma cell lines) for 2 h in a humidified 5% CO2 incubator at 37 °C. Donor-derived macrophages were harvested from plates using TrypLE Express (Life Technologies, Carlsbad, CA, USA) prior to the co-culture. Cell lines were fluorescently
labeled with cell trace violet (CTV) according to the manufacturer’s instructions. Where indicated, cancer cells were pretreated with 1 μg/mL (MCL and DLBCL) or 10 μg/mL (carcinomas) of mouse IgG1 anti-CD24 (CD24-mIgG1) (Clone SN3), IgG2a anti-CD24 (Clone ML5), 10 ng/mL of rituximab (Truxima), 10 μg/mL recombinant human Siglec-10 (rh Siglec-10) protein (Abcam), 10 μg/mL of mouse IgG1 anti-CD19 (Clone HIB19) and the same concentrations of appropriate isotype controls. For some experiments, macrophages were pretreated with 100 μg/mL of Fc blocker solution (IVIG, nanogam, Sanquin) for 40 min at 4 °C or 10 μg/mL of anti-Siglec-10 mAb (Clone 5G6, Biolegend). After co-culture, phagocytosis samples were stained with anti-CD11b-FITC or -APC (Immunotools) to identify human macrophages. Samples were analyzed by flow cytometry. Phagocytosis was measured as the number of CD11b+/CTV+ macrophages, quantified as a percentage of the total CD11b+ macrophages. The total number of remaining cancer cells was counted using flow cytometry and counting beads (Biolegend). Each phagocytosis assay (independent donor and experimental group) was performed in a minimum of a technical triplicate.

Statistics
The effect of the different antibodies on phagocytosis performed by different donors was determined by paired Student’s t-test. A comparison of three or more variables was analyzed with one-way ANOVA followed by the post-hoc Tukey test. All tests were performed using GraphPad Prism (GraphPad Prism; GraphPad Software, La Jolla, CA, USA). Where indicated, * = p < 0.05; ** = p < 0.01; *** = p < 0.001.
INVESTIGATIONS INTO CD24/SIGLEC-10 AXIS

References

Supplementary Material

Supplementary Figure 1. Production of CD24xCD19. (A) Schematic construction of the plasmid for lentiviral production inserted by a CD24-directed scFv antibody fragment and a CD19-directed scFv antibody fragment. (B) Production efficiency of CD24Xd19 harvested at different time points, checked by HA primary antibody (WB). (C) Non-reducing SDS monitoring, detected by Coomassie Brilliant Blue staining. (D) Concentration determination of CD24xCD19, as compared with known concentrations of HA-tagged antibodies (Dot plot).

Supplementary Figure 2. Percentage of M2c CD34-derived macrophage phagocytosis (WT and SIGLEC10 KO) after 2h co-culture with UPN-1 and HBL-2 cells treated with 1 µg/mL of CD24 mAb (SN3) or 25 µg/mL of CD24xCD19 construct.
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