Epitope mapping, expression and post-translational modifications of two isoforms of CD33 (CD33M and CD33m) on lymphoid and myeloid human cells

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We have tested the usefulness of several commercial anti-CD33 monoclonal antibodies (mAb) to determine the expression and localization of the two CD33 isoforms on several hematopoietic cell lines. The expression of the isoform CD33m, a CD33 transmembrane splice variant lacking the ligand-binding V immunoglobulin (Ig)-like domain, was detected by RT–polymerase chain reaction, western blot, confocal microscopy and flow cytometry on the membrane of several human cell types. CD33m was only detected by the anti-CD33 mAb HIM3-4 on the cell surface, whereas WMS53, P67.6, 4D3, HIM3-4, WM54, D3HL60.251 or MY9 detected the CD33M isoform, indicating that HIM3-4 is the only mAb recognizing CD33 C2 Ig domain. Accordingly, HIM3-4 binding to CD33 did not interfere with the binding of other antibodies against the CD33 V-domain. P67.6 mAb interfered with recognition by the rest of antibodies specific for the V domain. HIM3-4 staining could be increased after the sialidase treatment of all CD33+ cells. However, this increase was stronger in activated T cells, suggesting a CD33 masking state in this cell population. Confocal microscopy analysis of CD33m HEK 293T-transfected cells revealed that this protein is expressed on the cell membrane and also detected in the Golgi compartment. CD33 is constitutively located outside the lipid raft domains, whereas cross-linked CD33 is highly recruited to this signaling platform. The unique ability of HIM3-4 mAb to detect the masking state of CD33 on different cell lineages makes it a good tool to improve the knowledge of the biological role of this sialic acid-binding Ig-like lectin.

Keywords: anti-CD33 mAb / CD33 epitopes / CD33m isoform

Introduction

CD33 is a member of the sialic acid-binding immunoglobulin (Ig)-like lectin (siglec) family abundantly expressed on hematopoietic cells. Sixteen human siglecs have been identified so far, all containing one N-terminal V-set Ig-like domain followed by a variable number of C2 set domains in their extra-cellular regions (Crocker et al. 2007; McMillan and Crocker 2008; Von Gunten and Bochner 2008). The sialic acid-binding region is located on the V-set Ig-like domain, which contains two aromatic residues and one arginine motif highly conserved in all siglecs (May et al. 1998; Crocker et al. 1999; Crocker and Varki 2001). Since cell membranes are rich in sialic acids, ligand binding of siglecs can occur in cis and in trans, both affecting their functional properties. Thus, cis interaction can produce a masking state of the implicated receptors. CD22 and the majority of CD33-related siglecs contain one or more immunoreceptor tyrosine-based inhibitory motif (ITIM) sequences in their cytoplasmic tails, which enable them as putative inhibitory receptors that might contribute to the regulation of immune functions (Crocker et al. 2007; McMillan and Crocker 2008; Von Gunten and Bochner 2008). Recently, like with other regulatory receptor families, activating siglecs have been described, such as siglec-14 up to siglec-16.

CD33 (siglec-3) was cloned several years ago (Peiper et al. 1988; Simmons and Seed 1988). It contains two Ig-like extracellular domains, one ITIM and one ITIM-like motif in its cytoplasmic domain (Crocker et al. 2007; McMillan and Crocker 2008; Von Gunten and Bochner 2008). When phosphorylated, CD33 recruits protein tyrosine phosphatases SHP-1 and SHP-2 (Taylor et al. 1999; Ulyanova et al. 1999; Paul et al. 2000) and acts as an inhibitory receptor by ligation with CD64 on myeloid cells (Taylor et al. 1999). Anti-CD33 monoclonal antibodies prevent the generation of dendritic cells from monocytes and myeloid CD34+ precursors by inducing apoptosis (Ferlazzo et al. 2000; Vitale et al. 2001). Moreover, a role of protein kinase C in the serine phosphorylation of CD33 and its effect on lectin activity on myeloid cells (Grobe and Powell 2002), as well as a constitutive repressor activity of CD33 on human monocytes mediated by PI3K signaling (Lajaunias et al. 2005), have been described. Nonetheless, it remains largely unknown whether CD33 really...
functions as an inhibitory receptor as well as the ligand(s) recognized on their cellular targets.

Although CD33 has been currently described as a marker of immature and mature myeloid cells (Crocker et al. 2007; McMillan and Crocker 2008; Von Gunten and Bochner 2008), there is accumulated evidence of its expression on the subsets of B and activated T and natural killer (NK) cells (Handgretinger et al. 1993; Nakamura et al. 1994; Schmidt-Wolf et al. 1995; Dworzak et al. 1998; Eksioglu-Demiralp et al. 1999; Hernández-Caselles et al. 2006). Hence, it is highly expressed on myeloid committed cells of the bone marrow and circulating monocytes. CD33 expression is down-regulated to low levels on peripheral granulocytes and resident macrophages and it is constitutive on dendritic cells (Crocker et al. 2007; McMillan and Crocker 2008; Von Gunten and Bochner 2008). This expression pattern suggests a putative role of CD33 on myeloid differentiation and cellular function of monocyte and dendritic cells. Although some authors had reported its expression on certain lymphoid cells, this subject had not been definitively established. We have previously demonstrated that wide subsets of mitogen or alloantigen human activated T and NK cells do express the CD33 molecule at both protein and nucleic acid levels (Hernández-Caselles et al. 2006). Myeloid and lymphoid CD33 cDNA were identical, although lymphoid CD33 protein had lower molecular weight, suggesting cell type-specific post-translational modifications. Moreover, we also described a new isoform of CD33, named CD33m, generated by the alternative splicing of CD33 RNA, that could be expressed on all CD33 positive cells tested together with the higher molecule, named CD33M, corresponding to the currently known CD33. CD33m mRNA was mostly detected in natural killer cell line (NKL) and myeloid cell lines but it was poorly expressed in B cell lines and T lymphocytes (Hernández-Caselles et al. 2006). As depicted in Figure 1, the smaller isoform CD33m lacks the Ig-like V-type domain (the ligand-binding site), and the disulfide bond linking V and C2 domains of CD33M. Cross-linking of CD33 diminished the cytotoxic activity of NKL cells against K562 and P815 target cells, acting as an inhibitory receptor on NK cells (Hernández-Caselles et al. 2006). Thus, both isoforms could play a role in the regulation of both myeloid and lymphoid cells immune function.

CD33 is used as a diagnostic marker for acute myeloid leukemias (AMLs; Lo Coco et al. 2006). In this context, it is interesting to mention that a humanized form of P67.6 anti-CD33 mouse antibody conjugated with calicheamicin (named before CMA-676, gemtuzumab ozogamicin) was approved for therapy of conventional chemoresistant AML, taking the advantage of its property to undergo antibody-mediated endocytosis. Complete and partial remissions have been reported after this treatment (Lo Coco et al. 2006; Stasi et al. 2008). On the other hand, viral infections have been observed in patients treated with this approach (Yoshimi et al. 2008) indicating that the immunosuppression induced by gemtuzumab ozogamicin may affect other cells different from monocytes/macrophages or dendritic cells.

Herein, we have mapped the epitopes of both isoforms recognized by commercial anti-CD33 monoclonal antibody (mAb), trying to gain further insights into the regulatory role of these molecules that could be useful to improve their potential on basic science and clinical applications. We have found that P67.6, WMS3, WM54, 4D3, D3HL60.251 or MY9 mAb recognize an epitope located in the V domain, which is only expressed in CD33M, whereas HIM3-4 is the only commercial antibody among all assayed that recognizes the C2 domain, which is the only extracellular domain expressed in CD33m. Previous treatment with HIM3-4 did not affect binding of other anti-CD33 antibodies, although pre-incubation with antibodies specific for the V domain did interfere staining with HIM3-4. Furthermore, we show that HIM3-4 mAb could be used to detect the masked state of CD33 on different cell types, since sialidase treatment increases its specific staining on the cell membrane.

Regarding the expression of CD33 isoforms, we have demonstrated for the first time that both isoforms are expressed on transfected HEK 293T cells, on different lymphoid and myeloid human cell lineages as well as on monocytes, neutrophils and activated T lymphocytes obtained from the blood of healthy donors. Transfected cells revealed that CD33M and CD33m can be expressed both at the Golgi compartment and on the cell membrane. The expression of both CD33 isoforms as membrane proteins with different states of glycosylation reveals additional properties in the regulation of both myeloid and lymphoid immune function.

Results
CD33m can be detected by flow cytometry on the membrane of HEK 293T-transfected cells only by HIM3-4 anti-CD33 mAb

We have previously proved that two isoforms of CD33 mRNA, named as CD33M and CD33m, are expressed on
CD33 human leukocytes; although the new described isoform, CD33m could not be detected as a membrane protein. In the present work, we have tested several commercial anti-CD33 mAb, searching for tools that allow us to identify the CD33m isoform and to discriminate between both spliced variants. First of all, we transiently transfected the human cell line HEK 293T with a plasmid control (pcDNA3), or plasmids containing the cDNA encoding CD33M or CD33m proteins, in order to assess whether CD33m is expressed as a membrane protein. Expression of CD33 was then studied by flow cytometry 24 h later, by using a panel of seven commercial anti-CD33 mAb (clone WM53, P67.6, 4D3, HIM3-4, WM54, D3HL60.251 and MY9). As it can be observed in Figure 2, labeling with every anti-CD33 mAb used displayed high fluorescence intensity on CD33M-transfected cells, compared with cells transfected with empty vector pcDNA3 (H–N vs. A–G). Conversely, labeling CD33m-transfected cells with every anti-CD33 mAb tested, except HIM3-4, did not produce any significant difference compared with negative control cells (Figure 2O–Q and S–U vs. A–C and E–G). Notably, we observed that HIM3-4 was able to stain with a similar pattern both CD33M and CD33m-transfected cells (Figure 2K and R vs. D). These results indicate that epitopes recognized by WM53, P67.6, 4D3, WM54, D3HL60.251 and MY9 mAb are located in the V-Ig-like domain, whereas that recognized by HIM3-4 is located in the C2-like domain, which is common to both CD33 isoforms. Hence, from all the mAb tested, HIM3-4 is the only one able to detect the small CD33m isoform. However, since the C2-like domain is common to CD33M and CD33m, HIM3-4 is not able to distinguish between both expressed isoforms.

Epitope mapping analysis by anti-CD33 mAbs competition

To further map the epitopes recognized by different anti-CD33 mAb, binding competition experiments were performed. Thus, HEK 293T cells transfected with plasmids containing the CD33M encoding cDNA were incubated first with an unconjugated anti-CD33 mAb, washed and then stained with saturating levels of another fluorescein isothiocyanate (FITC)-conjugated or phycoerythrin (PE)-conjugated anti-CD33 mAb. Next, the fluorescence intensity was analyzed by flow cytometry and compared with the fluorescence intensity displayed by direct staining with the fluorochrome-conjugated anti-CD33 mAb. Binding of fluorochrome-conjugated mAb was unaffected by irrelevant isotype-matched controls (data not shown). Figure 3A shows that the binding of either FITC-conjugated WM54, D3HL60.251, MY9 or PE-conjugated P67.6, 4D3 and WM53 mAb was unaffected when cells were previously incubated with the unconjugated HIM3-4 mAb, whereas HIM3-4 did compete with itself. These results indicate and confirm that the epitopes recognized by those mAb are sufficiently distant, so that the binding of HIM3-4 cannot be interfered, i.e. they are located in different domains. In turn, Figure 3B shows that previous incubation of CD33M-transfected HEK 293T cells with unconjugated P67.6 mAb did not significantly alter the labeling with FITC-conjugated HIM3-4, whereas it partially interfered cell staining with FITC-conjugated WM54, DHL60.251 or PE-conjugated WM53 and 4D3, and it completely interfered cell labeling with FITC-conjugated MY9 and itself. These data indicate that anti-CD33 monoclonal antibodies that recognize epitopes located in the V-Ig-like domain bind to three unrelated sites. Thus, P67.6 and MY9 bind to the same epitope (herein named epitope 1), which interferes with the binding sites of the rest of mAb assayed. In turn, WM54, WM53 and DHL60.251 share a common binding site (named epitope 2), and finally the mAb 4D3 can recognize a different epitope (named epitope 3). Competition assays between WM53 and HIM3-4 with P67.6, the main anti-CD33 mAb used on clinical and basic research applications, were also performed using different human cell types. Thus, Figure 3C shows that pre-incubation with unconjugated WM53 mAb notably inhibited the labeling with P67.6-PE on both the cell line U937 and blood monocytes, which constitutively express high levels of CD33 cell-surface molecules. These results confirmed that P67.6 and WM53 antibody-binding sites (corresponding to epitopes 1 and 2) are closely located in the V-Ig-like domain of CD33 proteins. Figure 3C also shows that pre-incubation of U937 cells with HIM3-4 did not interfere labeling with P67.6-PE, whereas the same treatment slightly reduced P67.6-PE staining of blood monocytes. Therefore, contrary to what happened with the transfected and cultured cells lines, HIM3-4 and P67.6 mAb slightly competed for binding to CD33 molecules on blood monocytes. This fact could be explained by the existence of different post-translational modifications or CD33 interaction with other membrane components on these particular cells. We also studied antibody competition on cell lines and primary myeloid cells by previous incubation of cells with P67.6 mAb and then staining with HIM3-4-PE. In this last case, we found that P67.6 and HIM3-4 mAb competed for CD33 binding (data not shown) suggesting that binding of mAb to the distal V-like domain blocks the access of anti-CD33 mAb to the proximal C2 domain.

Treatment with sialidase notably increases HIM3-4 antibody labeling

To assess whether the accessibility of HIM3-4 could be dependent on the sialylation level of cell surface glycoproteins including the CD33 molecule, we treated different cell types with sialidase from Arthrobacter ureafaciens, then the cells were extensively washed and stained with WM53, HIM3-4 or P67.6 mAb. As shown in Figure 4A, sialidase treatment of CD33M- and CD33m-transfected HEK 293T cells neither altered staining with WM53 nor with HIM3-4 mAb. However, as it can be observed in Figure 4B, sialidase treatment notably increased HIM3-4 staining of NKL and U937 cells, whereas cell staining with WM53 mAb remained unchanged. Similar results were obtained with blood monocytes and neutrophils from several healthy donors (Figure 4C). Finally, Figure 4D shows that staining of untreated or sialidase-treated activated T lymphocytes with mAb P67.6 or WM53 resulted in similar percentages of CD33 positive cells (63.6 and 65.2%, respectively). HIM3-4 staining of this cell population was quite low, ranging from 2.5 to 13.8% (Hernández-Caselles et al. 2006). However, treatment with sialidase produced a high increase in HIM3-4.
positive cells up to 42.0%. Altogether, these results show that the reactivity pattern of HIM3-4 mAb is dependent on the cell sialylation status, whereas the corresponding to P67.6 or WM53 antibodies is not. A possible explanation for this effect is that binding of HIM3-4 to the CD33 C2 domain could be masked by CD33 cis interaction with sialic acids displayed by proximal receptors on the cell membrane, including other CD33 molecules (Varki and Angata 2006). These results support the hypothesis that CD33 is constitutively and/or differently masked on the membrane of activated T cells and other cell types, respectively, likely depending on the specific glycosylation status of each cell type. Although our results cannot clarify the role of the CD33m isoform or the C2 domain on the CD33 masking state, we believe that the HIM3-4 mAb could be a useful tool to detect and study the masking state of this siglec.

**CD33m protein is correctly expressed both on the cell membrane and at the Golgi compartment in HEK 293T-transfected cells**

To further study the cell expression of both CD33 isoforms, we performed confocal microscopy analysis of HEK 293T CD33-transfected cells. To this end, HEK 293T cells were grown onto cover slides and then transiently transfected with 0.3 mg of pcDNA3 plasmids containing CD33M or CD33m cDNA. After 24 h transfection, detergent-permeabilized and non-permeabilized cells were stained with FITC-conjugated HIM3-4 and Alexa Fluor 488-conjugated anti-mouse (Figure 5A) or PE-conjugated P67.6 and Alexa Fluor 568-conjugated anti-mouse (Figure 5B) antibodies, processed and analyzed by confocal microscopy. As it can be observed in Figure 5A, CD33M and CD33m proteins were homogeneously distributed on the membrane of non-permeabilized
cells, and also they appeared irregularly distributed inside permeabilized cells. CD33M-transfected cells marked with mAb P67.6 (Figure 5B) displayed a similar staining pattern to that obtained with HIM3-4. However, CD33m-transfected cells were not stained by P67.6 mAb, which is consistent with results obtained by flow cytometry, since this antibody does not recognize the C2 domain of CD33 (Figure 5B). Next, to further explore the intracellular location of CD33M and CD33m, colocalization of CD33 with the cis-Golgi marker Rab1-EGFP (enhanced green fluorescent protein) was analyzed in permeabilized cotransfected HEK 293T cells (Pérez-Oliva et al. 2009). Figure 5C shows that both CD33 isoforms were highly concentrated at the Golgi compartment, indicating a similar processing mechanism of both proteins.

Altogether, these data indicate that the lack of the V domain in the CD33m molecule does not imply the modulation of its intracellular traffic and location.

**CD33M and CD33m are detected by western blot in both transfected HEK 293T and several hematopoietic cells**

Further demonstration of dual expression of CD33M and CD33m as transmembrane proteins in transfected cells HEK 293T, as well as in several hematopoietic cell lineages, was achieved by western blot analysis using the polyclonal anti-CD33 Ab, H-110. Since H-110 antibody binds to an epitope located in the CD33 cytoplasmic tail, it identifies both CD33 isoforms. Thus, western blotting of CD33M-transfected,
CD33m-transfected or CD33 (M+m)-cotransfected HEK 293T cells revealed the CD33M bands at $\approx 53–55$ kDa (Figure 6A), which is consistent with our previous immunoprecipitation data obtained with the WM53 anti-CD33 mAb (Hernández-Caselles et al. 2006), and the CD33m protein bands at $\approx 32–33$ kDa (Figure 6A). These results confirmed that H-110 Ab recognizes the CD33m protein. Furthermore, we treated cell lysates with the glycosidase PNGaseF, which dissociates N-linked sugar chains from polypeptides, in order to check the glycosylation status of CD33 in this cell line. The results revealed the decreased molecular mass of both CD33 isoforms after PNGaseF treatment (Figure 6A), indicating that both of them are glycosylated when expressed on HEK 293T cells. Moreover, it could be observed that the molecular weight of the deglycosylated CD33M isoform was very close to the untreated CD33m isoform.

The expression level of CD33M and CD33m proteins was also tested in several leukemia human cell lines (Figure 6B). The lymphoblastoid Epstein–Barr virus-transformed cell line GUS was used as a CD33-negative control cell line (Hernández-Caselles et al. 2006). We identified from THP-1, HL-60 and U937 myeloid cell lines a wide band at the expected size of $75–77$ kDa (Figure 6B), and a smaller band of $\approx 37–38$ kDa. These bands might correspond to both isoforms of the CD33 antigen, being CD33M the heaviest and predominant isoform. The different mobility obtained by western blot from different cell lineages suggests that both proteins are heavily and differently glycosylated on myeloid cells. On both NKL cells and the erythroblastoid cell line K562, a CD33M band of 67 kDa could also be detected, as well as the small protein at 38 kDa, which might correspond to the CD33m isoform.
We also found the CD33M and CD33m bands (Figure 6C, lanes 1 and 2) on the cell lysates of purified monocytes obtained from the blood of two healthy donors, indicating a constitutive expression of CD33m in human monocytes. As expected, the corresponding bands were absent from CD33− resting primary lymphocytes (lane 3), although they were displayed from CD33+ polyclonal activated T lymphocytes (lane 4), repetitively stimulated by allogeneic stimulation in vitro (Hernández-Caselles et al. 2006).

Finally, we tried to discard the possibility that CD33 degradation products could be detected as the CD33m isoform. Taking advantage that CD33 can be ubiquitylated and degraded by the proteasome (Orr et al. 2007), we performed western blot analysis after cell treatment with the proteasome inhibitor lactacystin. Figure 6D shows that CD33M and CD33m bands from cell lysates of treated and untreated cells did not reveal differences regarding size or amount of the corresponding protein. Results from western blot experiments after cell treatment with the lysosomal inhibitor chloroquine did not show any CD33 degradation band from lysosomal proteolysis (data not shown). Altogether, these results exclude that any lower band assigned to the CD33m isoform could be attributed to CD33M proteolytic products. Our results also indicate that the predominant CD33M and the small CD33m isoform...
isoform may be always simultaneously expressed as proteins in all CD33+ cells of both myeloid and lymphoid lineages.

Quantitative measurements of CD33M and CD33m gene transcription by real-time RT–polymerase chain reaction

To further confirm that the bands depicted in Figure 6 correspond to the splice variants of CD33 and to explore the significance of CD33m mRNA in the cell lines used in this study, we determined the expression level of CD33M and CD33m mRNA by quantitative real-time RT–polymerase chain reaction (PCR) (Figure 7). We used HEK 293T as a negative control since these cells do not express the CD33 transcripts. Quantitative differences in CD33 expression between cell lineages were confirmed. Hence, myeloid U937 cells expressed the highest total CD33 mRNA, followed by erythromyeloid K562 cells, which expressed an intermediate level, and NKL cells that expressed the lowest relative level (Figure 7A). Quantitative analysis of both isoform mRNA expression showed that the relative levels of CD33M and CD33m were variable between cell lineages, being CD33m lower in U937 and similar in K562 and NKL (Figure 7B). The relative level of CD33M was higher in U937 and NKL than in K562, which displayed similar levels of mRNA of both isoforms. These data indicate that CD33m mRNA is expressed at a significant and measurable, although lower, level than CD33M mRNA in these cell lines.
CD33 resides constitutively outside the lipid-enriched microdomains in lymphoid and myeloid cell lineages, but it is highly colocalized with them after cross-linking with specific mAbs. Finally, since CD33 has been referred to as inhibitory receptor, we planned to study the colocalization of CD33 (using unconjugated anti-CD33 WM53 or HIM3-4 as primary antibodies and a secondary goat anti-mouse Alexa Fluor 568 Ab) and lipid raft microdomains [revealed by staining with cholera toxin B (CTXB)-Alexa Fluor 488] on the membrane of activated polyclonal T cells, HL-60 and NKL leukemic cells (Figure 8). Confocal analysis revealed a similar pattern of CD33 membrane distribution on all of these cells, i.e. small patches distributed all around the cell membrane.

These results were compared with those obtained by colocalization analysis of CD71 and NKG2D as negative and positive controls of colocalization with lipid rafts, respectively (Harder et al. 1998; Endt et al. 2007), on NKL cells. Our results showed that CD33 was constitutively located outside the lipid rafts on activated T lymphocytes and HL-60 myeloid cells (mean of the Pearson coefficient was 0.104 ± 0.010 and 0.187 ± 0.024, respectively; Figure 8A). Similarly, CD33 did not colocalize with membrane rafts on NKL cells, presenting a mean of the Pearson coefficient (0.115 ± 0.013 when using WM53 mAb and 0.097 ± 0.055 when using HIM3-4 mAb; Figure 8A) alike to CD71 molecules (0.110 ± 0.013), whereas NKG2D strongly colocalized with lipid rafts (0.529 ± 0.023; Figure 8A).

Next, we determined the partitioning of cross-linked CD33 and NKG2D with lipid rafts on NKL cells using the corresponding primary antibody for 20 min at room temperature, then incubation with Alexa Fluor 568 goat anti-mouse secondary antibody for 20 min at 37°C, and finally, NKL cells were fixed with paraformaldehyde and stained with CTXB-Alexa Fluor 488. Our results showed (Figure 8B) that cross-linked CD33 highly colocalizes with glycolipid-enriched microdomains (GEMs), reaching a coefficient of colocalization with GEMs similar to that of the NK cell activating receptor NKG2D (Endt et al. 2007).

Discussion

Anti-CD33 mAb have been proved to be useful agents for CD33 basic research and for the diagnostic and immunotherapy of AML and acute promyelocytic leukemia (APL). Randomized trials evaluating the addition of humanized anti-CD33 mAb such as gemtuzumab ozogamicin (hp67.6 mAb conjugated with calicheamicin) and lintuzumab (huM195 mAb conjugated with gelonin) to the standard induction therapy and conventional chemotherapy of relapsed AML and APL are ongoing or pending of results (Döhner et al. 2010). However, most of the biochemical and functional characteristics of anti-CD33 mAb, as long as the expression and function of CD33 molecules remain largely unknown. In this context, our group has earlier described a new smaller CD33 isoform named CD33m, which is produced by alternative splicing of the CD33 gene (Hernández-Caselles et al. 2006). It lacks the V-Ig-like domain that interacts with sialic acids, but conserves the C2-Ig-like and the intracellular domains. In our previous work, we also detected a constitutive mRNA expression of CD33m on myeloid leukemic cell lines, and an induced expression on NKL cells and polyclonal-activated human T lymphocytes, by RT–PCR and northern blot assays. The CD33m extracellular portion was successfully expressed as a soluble fusion protein on transfected human cells, suggesting that it could play a potential role as a membrane receptor. However, we were unable to elucidate whether CD33m protein could be expressed on the membrane of those cells, since the assayed anti-CD33 mAb did not distinguish between both isoforms.

According to the above statements, our goals here were to know whether commercial Ab could distinguish between CD33 isoforms and to study the putative expression of CD33m protein on the membrane of CD33+ cells. These findings would enable us to further explore the cellular expression and biological function of CD33m. Our results have demonstrated that CD33m is expressed on the membrane of...
transfected cells, suggesting that the physiological expression of CD33m is feasible. Furthermore, by using this CD33m cell-transfected model, we have found that HIM3-4 is the only commercial antibody that recognizes the small isoform CD33m, as it binds to the C2-Ig-like domain of this molecule. Albeit, given that the C2 domain is common to both isoforms, it cannot discriminate between CD33M and CD33m proteins, indicating the absence of tools to distinguish CD33m and CD33M isoforms on the cell surface. We have also found here that other antibodies available for CD33 analysis, including P67.6, recognize epitopes located in the V domain of the molecule, which is contained only in CD33M. Moreover, M195 mAb, the murine Ab portion of lintuzumab, cross-blocked the specific binding of MY9 mAb (Scheinberg et al. 1989), indicating that M195 also recognizes the CD33 V-type Ig domain. Hence, all studies performed with these anti-CD33 mAb only detected biological functions mediated by the higher CD33 isoform. Additionally, HIM3-4 binding to CD33 expressed on transfected or leukemic cell lines did not affect binding of other V domain-specific antibodies. Contrarily, previous treatment with mAb specific for the V domain of CD33 notably decreased access of HIM3-4 to the C2 domain, probably by sterical hindrance or by masking. On this regard, we have also found that unlike P67.6 and WM53, CD33 staining with HIM3-4 increased notably on all hematopoietic CD33-positive cells tested (T lymphocytes, monocytes, transfected cells, suggesting that the physiological expression of CD33m is feasible. Furthermore, by using this CD33m cell-transfected model, we have found that HIM3-4 is the only commercial antibody that recognizes the small isoform CD33m, as it binds to the C2-Ig-like domain of this molecule. Albeit, given that the C2 domain is common to both isoforms, it cannot discriminate between CD33M and CD33m proteins, indicating the absence of tools to distinguish CD33m and CD33M isoforms on the cell surface. We have also found here that other antibodies available for CD33 analysis, including P67.6, recognize epitopes located in the V domain of the molecule, which is contained only in CD33M. Moreover, M195 mAb, the murine Ab portion of lintuzumab, cross-blocked the specific binding of MY9 mAb (Scheinberg et al. 1989), indicating that M195 also recognizes the CD33 V-type Ig domain. Hence, all studies performed with these anti-CD33 mAb only detected biological functions mediated by the higher CD33 isoform. Additionally, HIM3-4 binding to CD33 expressed on transfected or leukemic cell lines did not affect binding of other V domain-specific antibodies. Contrarily, previous treatment with mAb specific for the V domain of CD33 notably decreased access of HIM3-4 to the C2 domain, probably by sterical hindrance or by masking. On this regard, we have also found that unlike P67.6 and WM53, CD33 staining with HIM3-4 increased notably on all hematopoietic CD33-positive cells tested (T lymphocytes, monocytes, transfected cells, suggesting that the physiological expression of CD33m is feasible. Furthermore, by using this CD33m cell-transfected model, we have found that HIM3-4 is the only commercial antibody that recognizes the small isoform CD33m, as it binds to the C2-Ig-like domain of this molecule. Albeit, given that the C2 domain is common to both isoforms, it cannot discriminate between CD33M and CD33m proteins, indicating the absence of tools to distinguish CD33m and CD33M isoforms on the cell surface. We have also found here that other antibodies available for CD33 analysis, including P67.6, recognize epitopes located in the V domain of the molecule, which is contained only in CD33M. Moreover, M195 mAb, the murine Ab portion of lintuzumab, cross-blocked the specific binding of MY9 mAb (Scheinberg et al. 1989), indicating that M195 also recognizes the CD33 V-type Ig domain. Hence, all studies performed with these anti-CD33 mAb only detected biological functions mediated by the higher CD33 isoform. Additionally, HIM3-4 binding to CD33 expressed on transfected or leukemic cell lines did not affect binding of other V domain-specific antibodies. Contrarily, previous treatment with mAb specific for the V domain of CD33 notably decreased access of HIM3-4 to the C2 domain, probably by sterical hindrance or by masking. On this regard, we have also found that unlike P67.6 and WM53, CD33 staining with HIM3-4 increased notably on all hematopoietic CD33-positive cells tested (T lymphocytes, monocytes,
neutrophils and CD33+ cell lines) after the elimination of sialic acids by treatment with sialidase. These results indicate that differences in glycosylation can affect the HIM3-4 recognition of the CD33M and CD33m C2 domains in two manners: (i) glycan density might prevent the binding of HIM3-4 mAb to its epitope by steric or electrostatic impediments or (ii) the HIM3-4 epitope might be partially masked on the cell membrane, because of the interaction in cis of CD33M with nearby sialylated receptors. We believe that the second possibility is the most likely option, since HIM3-4 is able to strongly stain highly glycosylated CD33M molecules of 75 kDa molecular weight expressed on positive cell lines such as U937 or HL-60, whereas the same antibody poorly stained activated T lymphocytes, which express a 67 kDa molecular weight CD33M protein and it probably is less glycosylated. According to others, the interaction of siglecs with ligands in cis or trans can have important implications on their biological function (Varki and Angata 2006).

Ligands expressed on the same cell surface may bind to siglecs in steady state, setting a threshold that effectively competes with the interaction with ligands expressed on the membrane of other cells. Another possibility is that the interaction of siglecs with ligands in cis is a constitutive siglec state to monitor the status of sialylation of the cells that express them (Varki and Angata 2006). Thus, the occurrence, mechanism and biological relevance of masking phenomenon require further investigation. Our results also suggest that HIM3-4 mAb could be very useful as a tool to detect and study the CD33 masking state on the surface of CD33+ cells.

Confocal analysis of CD33-transfected cells has revealed that both CD33 isoforms are simultaneously expressed on the cell membrane and the intracellular Golgi compartment, as demonstrated by colocalization with Rab1-EGFP. These findings support the thought that the small CD33 isoform can play a role in maintaining the glycosylation status of the cell, allowing an equilibrium of CD33 expression.

Materials and methods
Reagents, cytokines and antibodies
Complete culture medium (CCM) was RPMI-1640 medium (Gibco, Life Technologies, Grand Island, NY) with glutamax-I (t-alanine-t-glutamine), 10% heat-inactivated fetal calf serum (FCS, Gibco, Life Technologies), 100 U/mL penicillin and 100 mg/mL streptomycin. When activated T lymphocytes were cultured, recombinant interleukin-2 (rIL-2; Hoffman-La Roche, Nutley, NJ) was used in a range between 50 and 100 U/mL.

The anti-human antibodies used in this work were: mouse purified or PE-conjugated IgG1 anti-CD33 (clone P67.6, Becton Dickinson, San José, CA), mouse purified or FITC-conjugated IgG1 anti-CD33 (clone HIM3-4, BD-Pharmingen, San Diego, CA), mouse PE- and FITC-conjugated IgG1 anti-CD33 (clone WM53, Becton Dickinson), mouse PE- and FITC-conjugated Ig2b anti-CD33 (clone 4D3, Caltag, Burlingame, CA), mouse FITC-conjugated IgG1 anti-CD33 (clone WM54, Santa Cruz Biotechnologies, CA), mouse FITC-conjugated IgG2b anti-CD33 (clone MY9, Beckman Coulter, Fullerton, CA), mouse FITC-conjugated IgG1 anti-CD33 (clone D3HL60.251, Immunotech SAS, Beckman Coulter company, Marseille, France), mouse IgG1 anti CD-71 (clone OKT9, eBioscience, San Diego, CA), mouse IgG1 anti-NKG2D (clone 1D11, eBioscience) and rabbit polyclonal anti-CD33 (H-110, Santa Cruz Biotechnologies).

Expression constructs and transfection
Total RNA from cultured cells was obtained by using the Micro RNA Isolation Kit (Stratagene, La Jolla, CA) according to the protocol provided by the manufacturer. cDNA was synthesized from 1–2 μg of RNA using oligo(dT)12–18 and the SuperScript II RNase H reverse transcriptase, according to the protocol of Life Technologies and based on the human CD33 cDNA sequence (GenBank XM-057602). The following cellular and biochemical analysis of CD33 isoforms...
forward ATCAGTAAGCTTCTCGACATGGCCGCTGC and reverse CGATGCAATTTCAAGAGAAGAAAATTGGAGA CATG primers were used to amplify by RT–PCR, the complete open reading frame of CD33. PCR experiments were run with an annealing temperature of 68°C, 1.5 mM Mg²⁺ and Ecotaq DNA polymerase (5 U/mL, Ecogen, Barcelona, Spain). The above pair of primers amplified two fragments of 1392 and 983 bp, named CD33M and CD33m, respectively. The obtained PCR products were cloned in the cloning expression vector pcDNA3 (Invitrogen, Carlsbad, CA) under HindIII and EcoRI restriction sites.

Rab1 fused to the fluorescent protein EGFP in pEGFP-N1 vector was kindly provided by Dr. Garcia-Borrón, Department of Biochemistry and Molecular Biology, University of Murcia (Pérez-Óliva et al. 2009).

HEK 293T cells were grown in 6- or 12-well dishes using Dulbecco’s modified Eagle’s medium, supplemented with 10% FCS, 1% glutamine, 100 U/mL penicillin and 100 mg/mL streptomycin sulfate. Cells grown to 80% confluence were transfected with 0.6 or 0.3 µg plasmid DNA/well for 6- or 12-well plates, respectively, unless otherwise specified, and Lipofectamine 2000 according to the manufacturer’s instructions. For cotransfections, 0.3 or 0.15 mg of each plasmid was employed.

**Cells and cell lines**

Human myeloid cell lines U937, HL-60 and THP-1, the Epstein–Barr virus-transformed B cells GUS, the NK cell line NKL (kindly provided by Dr. M. Robertson, Boston, MA) and the K562 erythroleukemia were grown in CCM. Human peripheral blood lymphocytes (PBLs) were obtained from healthy volunteers and purified by discontinuous density gradient in Lymphoprep (Nycomed Pharma, Oslo, Norway) according to the standard protocols. The polyclonal T cells used in this work were generated by two cycle stimulation of peripheral blood lymphocytes (PBLs) with irradiated allogeneic LCL-GUS and allo- geneic human splenic cells as described (Hernández-Caselles et al. 2006). After 2–4 weeks culture in rIL-2 (50–100 U/mL) supplemented CCM, expanded T cells were phenotypically analyzed.

When indicated, cells were treated with sialidase from *A. ureafaciens* (Roche, Penzberg, Germany) 10–20 mU/10⁵ cells, in RPMI+4 (2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) 50 mM at pH 6.9 for 1.5 h at 37°C as described before (Razi and Varki 1998). Then, cells were washed with 2% FCS in PBS and stained with the corresponding mAb.

**Immunofluorescence staining and confocal microscopy**

Phenotypic analysis of cells was carried out by immunofluorescence on a FACSort cytomter (Becton Dickinson) after staining with the appropriated combination of fluorochrome-conjugated mAb as described previously (Hernández-Caselles et al. 2006). Flow cytometry data were analyzed by using CellQuest and Paint-a-Gate programs, all from Becton Dickinson. A minimum of 4000 events per sample were analyzed.

For confocal analysis, cells were adhered to 3% paraformaldehyde 2%, for 10 min. After washing with PBS/ Gly 100 mM, cells were stained by indirect immunofluorescence treating them with the corresponding primary antibody at 10 mg/mL for 15 min at room temperature, followed by an Alexa Fluor 568-conjugated goat F(ab′)2 anti-mouse secondary antibody (Molecular Probes, Invitrogen), for detection of different markers. The CTXB binds to lipid raft-enriched GM₁ ganglioside and has been widely exploited to visualize lipid rafts. Finally, the cells were stained with CTXB (Molecular Probes) conjugated to Alexa Fluor 488 at 10 mg/ mL for 10 min at room temperature as described (Rubio et al. 2004). The coverslips were then washed once and mounted with Dako Fluorescent Mounting Medium (Dako, Carpinteria, CA). Rab1 was detected by the intrinsic fluorescence of the fused EGFP moiety. Samples were examined with a Leica laser scanning confocal microscope (True confocal scanner TCS-SP2). For dual-color analysis, cells were excited at 488 and 568 nm, and fluorescence of Alexa Fluor 488 (green) and Alexa Fluor 568 (red) was detected sequentially to avoid cross-talk. All images represent Z-series pileups of several transverse slices acquired at 0.1–0.5 mm intervals from the top to the bottom of the cell. Confocal sections were obtained through an ×100 objective (numerical aperture 1.4).

Colocalization of pairs of cell surface molecules was determined by importing the digitized images to ImageJ in order to read and collect all intensity data pairs of the slices that form the three-dimensional arrays of the double-labeled cells. Then, files containing data pixels obtained from ImageJ were transferred to SPSS v11.0 for computation and statistic correlation analysis of two variables, red and green intensities, and was obtained the correlation coefficient or Pearson’s coefficient, indicative of the colocalization in the samples. (ImageJ is a public domain image processing program inspired by NIH Image that runs on any platform with Java. ImageJ and all required plugins can be freely downloaded from the web site http://rsb.info.nih.gov/ij/).

**Western blot analysis**

Cell pellets were resuspended in lysis buffer (50 mM Tris–HCl, pH 7.5, 150 mM NaCl, 1% NP-40, 1 mM phenylmethylsulfonyl fluoride) containing 2 µg/mL of protease inhibitors (leupeptin, aprotinin, chemostatin and pepstatin; Cell Signaling Technology®, Boston). After 20 min on ice, lysates were centrifuged at 12,500 rpm (15672 × g) for 15 min and diluted in electrophoresis sample buffer, boiled at 95°C for 5 min and separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS–PAGE). Finally, they were blotted onto polyvinylidene fluoride membranes, probed with the anti-CD33 rabbit polyclonal IgG antibody, H-110 and goat anti-rabbit horseradish peroxidase-conjugated antibody (Santa Cruz Biotechnologies) and developed using the enhanced chemiluminescence method (Amersham, Pharmacia Biotech).

**Lactacystin treatment**

Different cell lines were incubated with lactacystin 5 µM for 6 h in order to inhibit the proteasome activity. Controls cells were incubated with dimethyl sulfoxide since lactacystin was prepared in that solvent. Samples were lysed as previously described and western blot was carried out.
Endo-glycosidase digestion

Detergent-solubilized cell extracts were treated for 4 h at 37°C with 3 U of N-glycosidase F (PNGaseF) in 50 mM phosphate buffer, pH 7.0, containing 10 mM ethylenediaminetetraacetic acid and 0.1% sodium dodecyl sulfate (SDS). For PNGaseF digestion, samples were first denatured by heating at 95°C for 5 min. Digestion was stopped by addition of electrophoresis sample buffer.

Quantitative real-time RT–PCR

Total RNA was extracted from different cell lines with the GenElute mammalian total RNA Miniprep kit, following the manufacturer’s instructions. Total RNA was reverse-transcribed using SuperScript First-strand of Invitrogen, following the manufacturer’s instructions. Real-time RT–PCR was carried out by using primers that amplified ~100 bp fragments, one of them hybridizing at the boundary between two exons, to discard genomic amplifications. The PCR mix contained 2.25 μL of 100 μM primers, 12.5 μL of SYBR Green® PCR Master Mix, 1 μL of template cDNA and RNase free water to 25 μL. The reaction was performed in a 7500 Real-Time machine (Applied Biosystems, Foster City, CA, USA) under the following cycling conditions: 1 cycle at 95°C for 10 min, 40 cycles at 95°C for 15 s and at 60°C for 1 min. Fluorescence data were collected from each cycle and were analyzed by means of 7500 SDS Software (Applied Biosystems). The expression data of the genes were normalized to β-actin. The following primers were used for real-time RT–PCR:

(i) β-actin (forward, 5′-GATTACTGCTCTGGCTCTTAGC A-3′; reverse, 5′-GTCAGGAGGAGCAATGATCTT-3′);
(ii) qCD33Total (forward, 5′-CTCAGTGTGCTCATAATCACC CCAG-3′; reverse, 5′-GGTCTCTGGAACAGATGTC AGC-3′);
(iii) qCD33M (forward, 5′-GCAGGGCCCTGGCTATGGA TC -3′; reverse, 5′-CATGACCTCSTGGAGGAGCTTGCG-3′);
(iv) qCD33m (forward, 5′-CTGGGCAGACTTGACCCACA GGC-3′; reverse, 5′-GCAGCTGACAACCAGGAGAGT CG-3′).

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Conflict of interest

None declared.

Abbreviations

Ab, antibody; AML, acute myeloid leukemia; APL, acute promyelocytic leukemia; CCM, complete culture medium; CTXB, cholera toxin B; EGFP, enhanced green fluorescent protein; FCS, fetal calf serum; FITC, fluorescein isothiocyanate; GEMS, glycolipid-enriched microdomains; Ig, immunoglobulin; mL, recombinant interleukin; ITIM, immunoreceptor tyrosine-based inhibitory motif; LCL, lymphoblastoid cell line; mAb, monoclonal Ab; NK, natural killer; PBLs, peripheral blood lymphocytes; PCR, polymerase chain reaction; PE, phycoerythrin; siglec, sialic acid-binding immunoglobulin lectin.

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