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CD277 an Immune Regulator of T Cell Function and Tumor Cell Recognition

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1. Introduction

Molecules involved in tumor cell recognition are potentially important targets in cancer therapeutics. Therefore, research on the identification of tumor cell antigens as well as, immune-regulatory molecules is essential. The study of new co-stimulatory molecules has attracted special interest due to, their role in activation and/or inhibition of effector function of immune cells. In this chapter, we focused on the study of CD277 and its putative counter-receptor, and their possible role in activation or inhibition of immune cells and recognition of cancer cells.

CD277 has been involved in immune cells regulation, because of its role in activating or inhibiting effector, cytokine secretion and cytotoxic functions, depending on the activating conditions of these cells. Moreover, the fact that the stimulation of tumor cell lines with anti-CD277 leave to a better recognition and killing by $\gamma\delta$ and $\alpha\beta$ T cells lead us to postulate that these molecules is involved also in tumor recognition. We will discuss different aspects of CD277 and its counter-receptor in this chapter.

Classically, the immune response has been divided into innate and adaptive immunity, with distinct properties. The innate immune response uses a small number of receptors that detect a limited set of conserved antigens. Induction of the adaptive immune response involves antigen presenting cells (APC) and T cells (Borghesi & Milcarek, 2007). The interaction between the T cell and the APC is the pivotal step that controls a series of events, including T cell activation, cell division and effector differentiation (Zhu & Chen, 2009). However, recognition of the processed antigenic peptide coupled to Major

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Histocompatibility Complex (MHC) class I or II molecules on APC is not enough to activate T cells, they require an antigen independent second signal involving the B7 family molecules. The lack of this co-stimulatory signal induces anergy (Ledbetter *et al.*, 1990).

CD28 and the related molecule CTLA-4, together with their natural ligands CD80 and CD86 are the classical co-stimulatory molecules (Ward, 1996). CD28 is constitutively expressed on T cells and is involved in their activation through signal transduction. CTLA-4, which delivers a strong inhibitory signal, is expressed on the T cell surface after engagement of TCR. CD80 and CD86 belong to the B7 co-signalling receptors family, they are cell surface glycoproteins that are essential to modulate and tune the T cell receptor (TCR)-mediated activation of T lymphocytes (Moretta & Bottino, 2004). Other members of this family are PD1 and their ligands PDL-1 and PDL-2 with inhibitory properties on T cell activation and ICOS and ICOSL, involved in T cell activation (Moretta & Bottino, 2004; Rietz & Chen, 2004). Members of the related B7 family belong to the immunoglobulins superfamily (IgSF). They have two Ig extracellular domains and show similarity to the variable (V) and the constant (C) domains of Ig. Previous studies have shown that IgV-like domains of B7.1 and B7.2 share similarity with myelin oligodendrocyte glycoprotein (MOG), chicken blood group system protein (BG) and butyrophilin (BT). Thus, these five proteins depict a subfamily in the IgSF. MOG is expressed only in the central nervous system (CNS) as a component of the myelin sheath and is involved as autoantigen in the pathogenesis of multiple sclerosis; BG proteins are only expressed in chicken and are associated with immunological functions acting as strong adjuvants when used in co-immunization with other proteins and; BT is a glycoprotein that forms a major component of milk fat globule membrane and is expressed in mammary gland at the end of pregnancy and during lactation but its function remains unknown. Whereas MOG and BG have only one extracellular IgV-like domain, BT has a B7-“like” structure with an IgV-like and an IgC-like domain (Henry *et al.*, 1999). Furthermore, BT has an intracellular domain of 166 amino acids named B30.2 and is presumably involved in the regulation of intracellular superoxide concentrations (Henry *et al.*, 1997).

Interestingly, the human *bt* and *mog* genes map to the distal part of the MHC class I region and six additional BT members were identified in this region. The six genes could be divided in two groups called BT2 and BT3 and are named using three different nomenclatures. Within these groups, there are BT2.1 (also called BTF1 or BTN2A1], BT2.2 (also called BTF2 or BTN2A2], BT2.3 (also called BTF1 or BTN2A1], BT3.1 (also called BTF5 or BTN3A1], BT3.2 (also called BTF4 or BTN3A2], BT3.3 (also called BTF3 or BTN3A3]. The degree of identity between members of BT2 and BT3 groups is around 50 %, while identity is around 95% within the same sub-family. Among these molecules BT3.1 or CD277 has a wide tissue distribution and its ligands are expressed on T cells suggesting that it could play a role in immune functions (Compte *et al.*, 2004). Other members of this family have also been identified on chromosome 6 (BTNL2) or other chromosomes.

Expasy site annotates 15 butyrophilin-like genes in humans including BT and MOG. Butyrophilin proteins typically have a signal peptide, an IgV-like and IgC-like domain, and a transmembrane and cytoplasmic domain. In addition, they often possess a heptad repeat which is a 7-aa sequence encoded by a single exon. Many butyrophilin molecules also contain a 166 aa B30.2 domain in the cytoplasmic region also found in tripartite motif (TRIM) proteins and stonutoxin. The precise function of the B30.2 domain in butyrophilin remains unclear. The B30.2 domain is also present in the C terminal part of various types of proteins which N-terminal globular domain may either contain Ig fold (butyrophilin), a

RING finger domain or a leucine zipper. It has been recently found that the protein TRIM5 interacts with HIV via its B30.2 domain. Xanthine oxidase interacts with B30.2 and may indirectly regulate nitric oxid production.

In pathological settings, proteins possessing B30.2 domain have been reported in over exuberant inflammatory responses such as Familial Mediterranean Fever (FMF) which is caused by mutation in the B30.2 domain of pyrin/marenostrin. In addition, mutations within this domain in the MID1 protein are also associated to the Opitz syndrome.

Because the IgV-like domains of B7.1 and B7.2 are more similar to the IgV-like domain of butyrophilin than to any other sequence, Linsley et al. proposed that B7 and butyrophilin molecules might have evolved from a common ancestral gene to compose a subfamily within the Ig superfamily. It is unclear whether B7 and butyrophilin molecules share common functions in regulating immune responses (Linsley *et al.*, 1994).

CD277 is thought to be involved in immune response because their expression can be modulated by pro-inflammatory cytokines such as TNF- α and IFN- γ (Compte *et al.*, 2004). Stimulation of BTN3 molecules results in phosphorylation BTN3A3 molecules leading to the attenuation of proliferation and cytokine secretion in CD4⁺ and CD8⁺ T cells in a CD4⁺CD25⁺ independent manner, demonstrating the agonistic properties of BTN3 to mediate negative-signal transduction (Yamashiro *et al.*, 2010).

In order to expand our knowledge about the CD277 and their role in tumor cell recognition, we analyzed different aspects of stimulation of leukaemia cell lines and some strategies to be used in the identification of the CD277 counter-receptor.

2. CD277: Its role in immune regulation and tumor cell recognition

The CD277 molecule is expressed on $\alpha\beta$, $\gamma\delta$ T cells, B and NK lymphocytes, monocytes, dendritic cells and hematopoietic precursors as identified by mRNA expression and flow cytometry. Moreover, the CD277 surface expression is constitutive on endothelial cells and it is increased by pro-inflammatory cytokines such as TNF- α and IFN- γ , suggesting that these molecules might be involved in the early events of tissue damage and inflammation (Compte *et al.*, 2004). Even though there are some reports regarding the effects of CD277 on immune cells, little is known about the functions of the CD277 counter-receptor in leukaemia and tumor cells. Here, we report our findings on functional properties of this counter-receptor and propose some strategies to determine the identity of this molecule.

2.1 Engagement of CD277 regulates $\alpha\beta$ T cells functions

Two different monoclonal antibodies (mAb) recognizing CD277 have been reported, 1) BT3.1, obtained from mice immunized with the extracellular domain of BTN3A1 (Compte *et al.*, 2004) and, 2) 232-5, obtained from mice immunized with the extracellular domain of BTN3A3 (Yamashiro *et al.*, 2010). In both cases, no differences in the recognition of the different isoforms of CD277 have been found. Therefore, these antibodies were used for the study of the immunomodulatory functions of CD277 on T cells. Yamashiro *et al.*, have evaluated the proliferation of CD4⁺ and CD8⁺ T cells populations in PBMC stimulated with anti-CD3 and anti-CD277 mAb. Interestingly, they found that the proliferation of both CD4⁺ and CD8⁺ T cells were suppressed by 232-5 but not by BT3.1 mAb. Moreover, IFN- γ and IL-4 production in CD4 T cells and IFN- γ in CD8⁺ T cells were down-regulated by 232-5 but not by BT3.1 mAb. Additionally, they report that this effect requires the cross-link of CD277 in the cell surface because the observed effects with the 232-5 mAb were lost when the Fab fragment was used (Yamashiro *et al.*, 2010).

These results suggest the negative effect of CD277 on T cell activation, but considering that the affinity of the BT3.1 mAb is lower than those observed with the 232-5 mAb and that the BT3.1 mAb did not show any effect on the T cell population analyzed it is possible that different effects could be attributed to CD277 on $\alpha\beta$ T cells. Using another mAb (20.1) we found that CD277 engagement enhanced the CD3 or CD3/CD28 co-stimulation at the proliferation and cytokine production levels. The effect of this mAb was associated with an increased intracellular signalling (Messal *et al.*, submitted).

On the other hand, T cells expressing $\gamma\delta$ TCRs have emerged as an important component of the immune repertoire and are included as component of the innate immune response (Nedellec *et al.*, 2010; Saenz *et al.*, 2010). In humans, the $\gamma\delta$ T cells are also located in peripheral organs and peripheral blood. Circulating $\gamma\delta$ T cells express a TCR comprising mainly V δ 2 and V γ 9 and cells with this phenotype represent 2% of mononuclear cells. These V γ 9V δ 2 T cells are activated by TCR stimulation with small non peptidic phosphorylated compounds also referred as phosphoantigens (Tanaka *et al.*, 1995). Unlike the conventional T cells, their activation does not require activating co-signals provided by CD28 - CD80 and CD86 interaction. However, their activation can be regulated by NKR (Das *et al.*, 2001; Halary *et al.*, 1997) and TLR (Deetz *et al.*, 2006; Wesch *et al.*, 2006). Moreover, engagement of NKG2D can activate them, without TCR stimulation (Rincon-Orozco *et al.*, 2005). Thus, considering the role of $\gamma\delta$ T cells in the anti-tumoral immune response (Tokuyama *et al.*, 2008) it is important to analyze the effects of CD277 on the functions of $\gamma\delta$ T cells as well.

2.2 CD277 and dendritic cells

We have reported that CD277 is expressed on the surface of resting and activated monocytes and monocyte-derived dendritic cells (iDC). The effect of CD277 on monocytes and iDC was analyzed in freshly isolated monocytes and monocyte-derived dendritic cells stimulated with anti-CD19 or anti-CD277 (anti-BT3.1). We found that upon stimulation for 24 h, anti-CD277 triggered the activation of monocytes, measured by up-regulation of the expression of CD86 on the cell surface as compared to cells treated with isotype-matched control. No effect was observed when control antibody (anti-CD19) or soluble anti-CD277 were used, indicating that cross-linking is required for the cellular activation observed (Simone *et al.*, 2010). Further, these results demonstrate that receptors for the Fc fragment of immunoglobulins (FcRs) dependent signalling are not responsible for cell activation.

On the other hand, a wide variety of receptors regulate the activity of cells of the myeloid lineage. We have also analyzed the effects of CD277 as co-receptor in proinflammatory responses triggered by pathogenic stimuli by stimulating monocytes and iDC with anti-CD277 in presence of different doses of Toll-like receptors (TLR) ligands (LPS and R-848). We observed that secretion of proinflammatory cytokines (IL8/CXCL8, IL-1 β and IL-12/p70) are increased in monocytes cultured with both anti-CD277 and TLR ligands compared with individual stimuli. Parallel experiments performed with iDC shown that cytokine secretion is similar to those observed with monocytes (Simone *et al.*, 2010).

These data suggest a possible role of CD277 as activation receptor on monocytes and dendritic cells. The need of cross-linking to obtain a biological effect on both monocytes and iDC is consistent with their possible agonist nature.

These data suggest a possible role of CD277 molecules as activation receptors on monocytes and dendritic cells. In addition, the need of cross-linking to obtain a biological effect on both monocytes and iDC is consistent with their possible agonist nature.

2.3 CD277 and tumor recognition

The effect of CD277 stimulation on $\alpha\beta$ T cells and monocytes and iDC described above, suggests the regulatory role of CD277, by activating or inhibiting the effector functions of immune cells. Moreover, the presence of the putative CD277 counter-receptor in the immune environment may play a critical role in defining where and when CD277 is engaged. We have reported that leukaemia cell lines and solid tumor cells lines such as HeLa and MCF-7, Raji, C91, HUT78 and JA16 (Compte *et al.*, 2004) express both CD277 and the CD277 counter-receptor. In addition, we have recently found (Harly *et al.*, in preparation) that Raji cells stimulated with anti-CD277 are better recognized and killed by V γ 9V δ 2 T cells. We postulated that the effect of CD277 stimulation on Raji cells would stand at the level of regulation of the antigen stimulation of V γ 9V δ 2 T cells and/or over-expression of the counter-receptor for CD277, thus, we investigated the effect of CD277 stimulation on the expression of CD277 counter-receptor in Raji cells. We used these cells because they express low levels of CD277 counter-receptor on the cell surface, therefore its regulation can be followed up by flow cytometry using the CD277-Fc fusion protein.

To analyze the expression of CD277 on different cell lines after treatment with anti-CD277, anti-CD80 or the Fab portion of anti-CD277 monoclonal antibodies, Raji, C91, TF1 or HUT78 cells were plated onto 96 round bottom wells (10^6 cells/well) and incubated with or without these antibodies at different concentrations (from 10 to 0.078125 $\mu\text{g}/\text{mL}$) for 2 hours. Then cells were extensively washed on PBS-FCS and incubated with or without 15 $\mu\text{g}/\text{mL}$ of CD277-Fc fusion protein (Compte *et al.*, 2004) for 30 minutes at 4°C. After incubation, cells were washed twice on PBS-2% FCS and stained with 1/100 goat-anti-human-Fc-PE for 15 min at room temperature. After wash, the cells were fixed with PBS - 1% formaldehyde and acquisition was done in a LSRII (BD bioscience). Data were analyzed with flow-jo software.

We found that the expression of CD277 counter-receptor is weak on non-stimulated Raji cells (Figure 1). However, the stimulation of Raji cells with anti-CD277 antibodies enhances the expression of CD277 counter-receptor compared to non-stimulated or stimulated with anti-CD80 antibodies controls (Figure 1A, B and C). These data suggest that up-regulation in the expression of CD277 counter-receptor is due to the CD277 engagement, because Raji cells stimulated with anti-CD80 express the counter-receptor at similar levels as non-stimulated cells non-stimulated (Figure 1B).

This effect was also observed on different cell lines such as C91, HUT78 and TF1. Although different basal levels of CD277 counter-receptor expression was found in these cells, in all cases stimulation with anti-CD277 antibodies lead to a higher expression of the counter-receptor on the cell surface (Figure 2). Additionally, stimulation with the Fab portion of the anti-CD277 has no effect on the expression of the counter-receptor; therefore it is necessary to cross-link CD277 on the cell surface to have the stimulatory effect observed (Figure 3). Moreover, the effect observed was dose-dependent, as shown in Figure 4.

2.4 Stimulation by CD277 and the regulation of the expression of CD277

We know that the expression of CD277 on immune cells is constitutive and that it is increased by proinflammatory cytokines such as IFN- γ and TNF- α (Compte *et al.*, 2004), however, little is known regarding the effect of CD277 stimulation on its own expression.

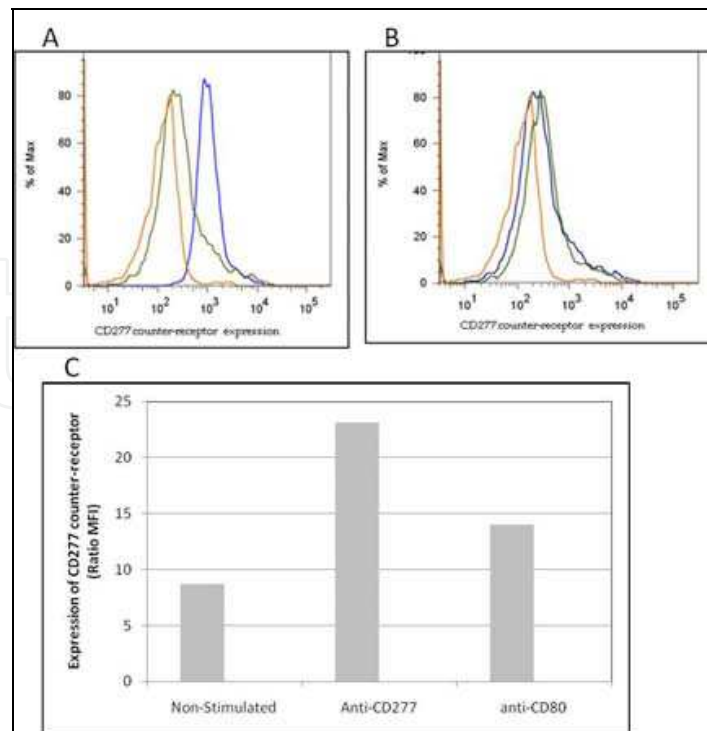


Fig. 1. Effect of CD277 stimulation in the CD277 counter-receptor expression on Raji cells. A) CD277 counter receptor expression in Raji cells after stimulation with anti-CD277 antibodies: Orange, basal fluorescence; green, expression of CD277 counter-receptor in non-stimulated and; blue, expression of CD277 counter-receptor in cells stimulated with anti-CD277 antibodies. B) CD277 counter-receptor expression in Raji cells after stimulation with anti CD80 antibodies(control): Orange, basal fluorescence , green, expression of CD277 counter-receptor in non-stimulated and; Blue, expression of CD277 counter-receptor in cells stimulated with anti-CD80 antibodies. C) MFI ratio was calculated by dividing the fluorescence intensity of cells in different conditions by the fluorescence intensity of the isotype control.

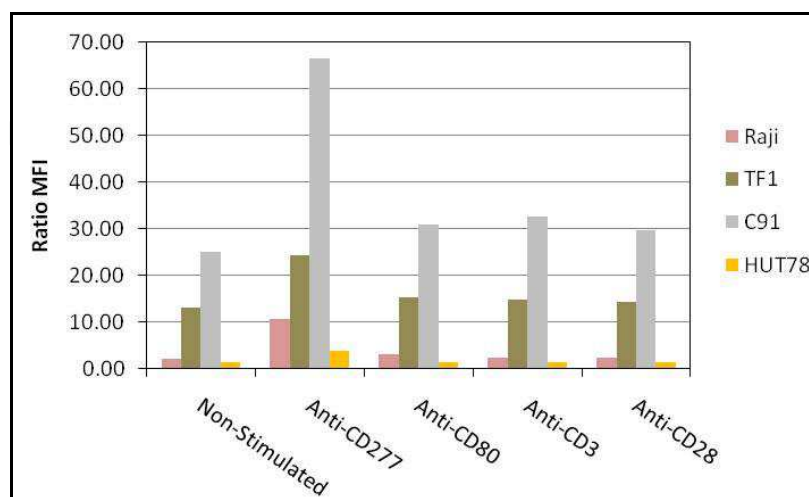


Fig. 2. CD277 counter-receptor expression on Raji, TF1, C91 and HUT78 cell lines stimulated by CD277. Each bar represents the mean fluorescence of triplicates divided by mean fluorescence of the isotype control.

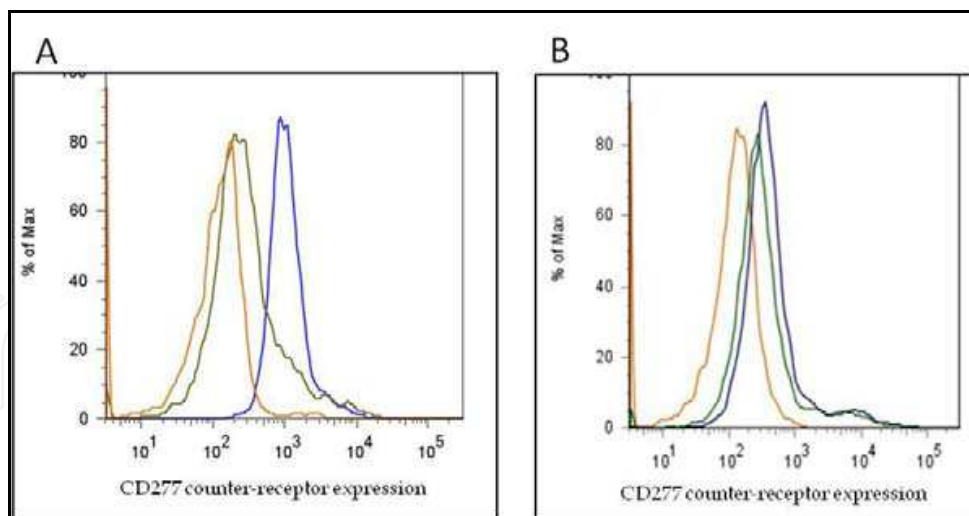


Fig. 3. Effect of CD277 cross-link on the CD277 counter-receptor expression in Raji cells. Orange, basal fluorescence; green, expression of CD277 counter-receptor in non-stimulated and Raji cells and; blue, expression in Raji cells stimulated with (A) anti-CD277 antibodies or (B) Fab of anti-CD277 antibodies.

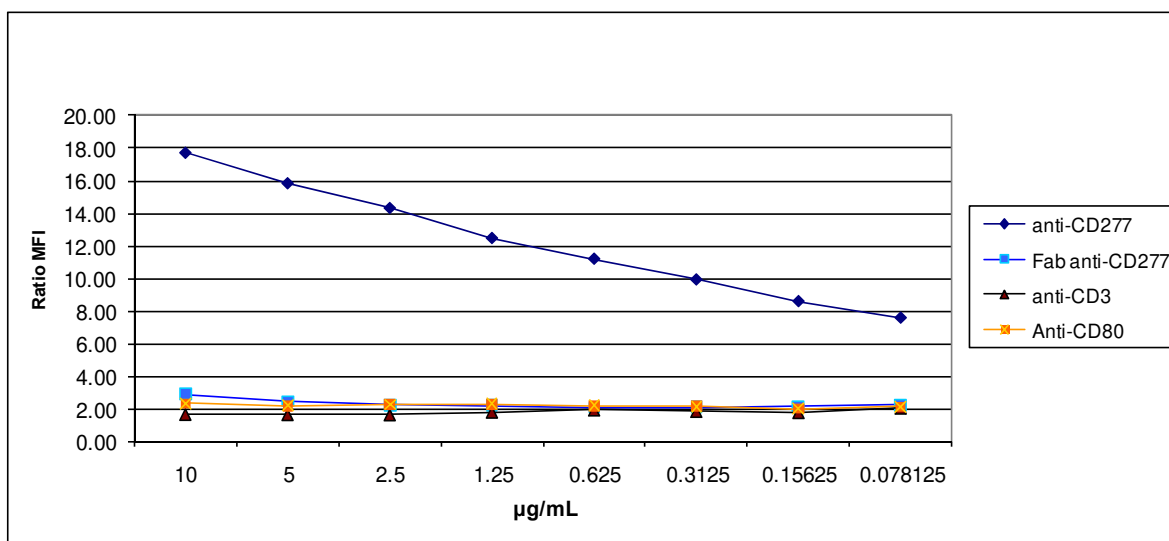


Fig. 4. Over-expression of CD277 counter-receptor in Raji cells is dose-dependent. Raji cells were tested with increasing concentrations of the anti-CD277, the Fab portion of the anti-CD277, anti-CD3 or anti-CD80 monoclonal antibodies and expression of CD277 counter-receptor was followed by flow cytometry.

To test this hypothesis, Raji cells (10×10^6) were cultured in RPMI 1640 + 10% FCS and stimulated with 10 µg/mL of either anti-CD277 or anti-CD80 monoclonal antibodies for 0, 2, 4, 6 or 24 hours at 37°C in a 5% CO₂ atmosphere. RNA was extracted with trizol® and RT-PCR using Taqman assays (Applied Biosystems) for CD277 isoforms (BTN3A1, BTN3A2, BTN3A3), and GAPDH, as endogenous housekeeping gene, was done. Expression of the CD277 isoforms was calculated as follows:

$$\Delta CT_{\text{stimulated}} = CT_{\text{Probe}} - CT_{\text{GAPDH}}$$

$$\Delta\Delta CT = \Delta CT_{\text{stimulated}} - \Delta CT_{\text{time} = 0}$$

Where CT_{Probe} is the CT for the CD277 isoforms, obtained from Raji cells stimulated with either anti-CD277 or anti-CD80, and $\Delta CT_{\text{time} = 0}$ is the ΔCT value for the CD277 isoforms at time = 0 hours (control).

The relative quantity (RQ) expression by using the formula:

$$RQ = 2^{-\Delta\Delta CT}$$

We found that the expression of BTN3A1 and BTN3A3 molecules but not BTN3A2, at 2, 4 and 6 hours is higher in Raji cells stimulated with anti-CD277 monoclonal antibodies and that this effect is lost after 24 hours (Figure 5). Taken together, our data suggest multiple functions of CD277: Promotion of the expression of CD277 counter-receptor and up-regulation of the expression of the CD277 molecules itself.

Although the CD277 monoclonal antibodies tested cannot differentiate between BTN3A1, BTN3A2 and BTN3A3 isoforms, we know that differences in the intracellular region of BTN3 molecules stand mainly with BTN3A2 which lacks the B30.2 domain. Therefore, since our data point out that signal transduction due to CD277 can be mediated by BTN3A1 and BTN3A3 isoforms, it seems likely that the B30.2 domain is involved this effect.

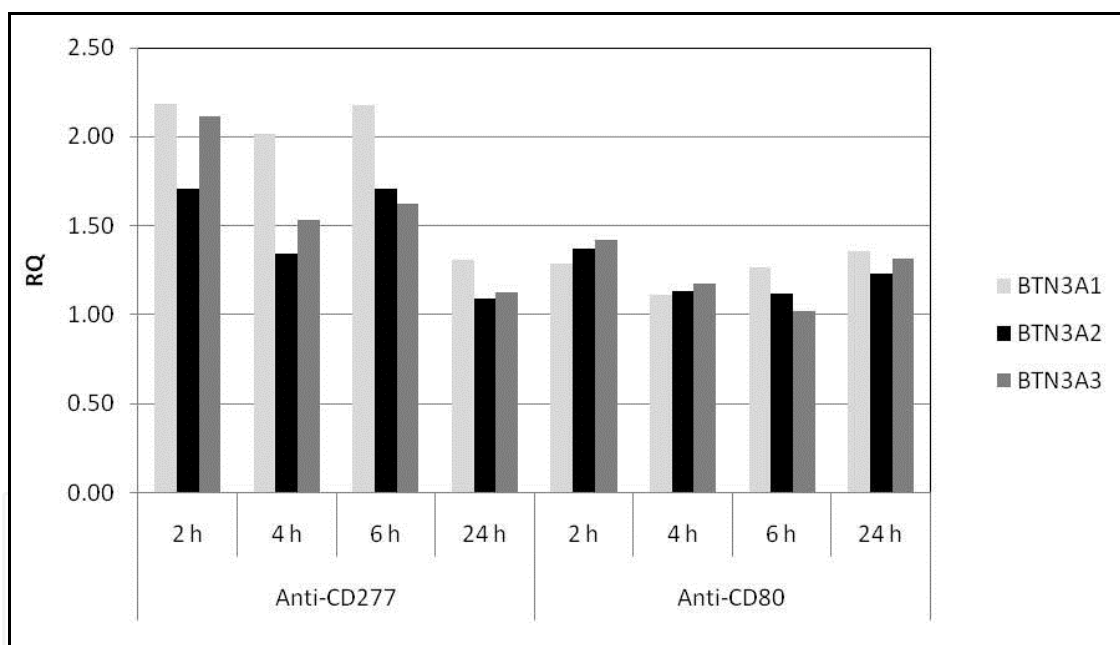


Fig. 5. A representative experiment on the gene expression of BTN3 isoforms on Raji cells stimulated by CD277. BTN3A1, A2 and A3 are the reported isoforms for CD277. The RQ was calculated as described. A RQ value higher than 2.0 was considered as indicative of gene over expression.

2.5 Effect of cytokines on the expression of CD277 counter receptor

It has been reported that cells stimulated by CD277 secrete inflammatory cytokines such as IFN- γ and TNF- α (Cubillos-Ruiz *et al.*, 2010; Yamashiro *et al.*, 2010) and that these cytokines may influence the expression of CD277. However, little is known about the modulation of the expression of the CD277 counter-receptor by these proinflammatory cytokines.

To further investigate the regulatory effects of inflammatory signals on the expression of CD277, C91 cells (50,000 cels/well) were treated with IFN- γ (5, 50, 500 and 5000 ng/mL), TNF- α (0.4, 4, 40 and 400 ng/mL), TGF- β (0.02, 0.2 and 2 ng/mL) and IL-22 (1, 10, 100 and 1000 ng/mL) for 24 hours at 37°C in a 5% CO₂ atmosphere. Following washes with PBS-FCS, the cells were incubated with or without (control) 15 μ g/mL of CD277-Fc fusion protein for 30 minutes at 4°C. After incubation, the cells were washed twice on PBS-2% FCS and stained with 1/100 goat-anti-human-Fc-PE for 15 min at room temperature. After wash, cells were fixed on PBS- 1% formaldehyde and expression of CD277 was assessed by flow cytometry (LSRII, BD bioscience). Analysis was done with flow-jo software. Our results show that stimulation with IFN- γ , TNF- α , TGF- β or IL-22 has no effect on the expression of CD277 counter-receptor in C91 cells (Figure 6).

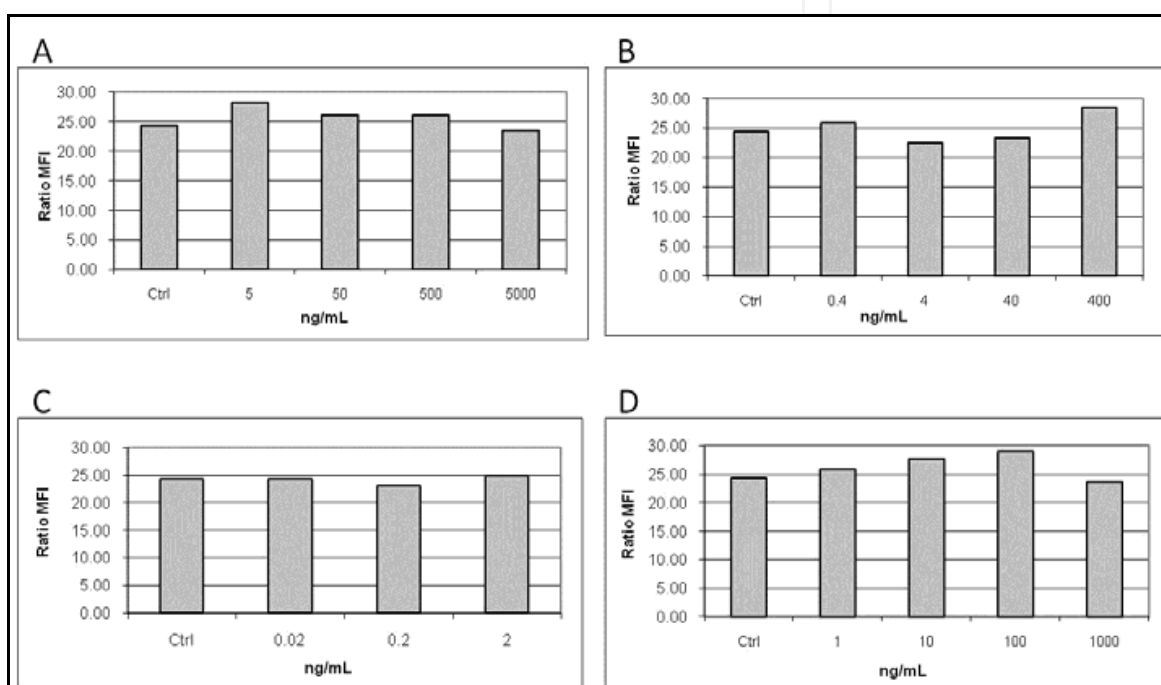


Fig. 6. A representative experiment on the expression of CD277 counter-receptor in C91 cells stimulated with different concentrations of (A) IFN- γ (B) TNF- α (C) TGF β and (D) IL-22 for 24 hours. Cells were stained with CD277-Fc fusion protein and analyzed by flow cytometry.

2.6 Strategies for CD277 counter receptor identification

The identity of CD277 counter-receptor is one of the main challenges in the near future, for a better understanding of the mechanisms involved in immune cell regulation and tumor cell recognition.

2.6.1 Methods to determine the CD277-counter receptor identity

In spite of the weak expression of the counter-receptor in different cell lines (Compte *et al.*, 2004), C91 cells were selected because of the expression profile is higher than in other cell lines such as Raji, TF1, HUT78 or JA16. In order to obtain a cell population with a stable expression of the CD277 counter-receptor, C91 cells were stained with the CD277-Fc fusion protein as described above. The positive population was identified by comparing to unstained control and the 1% most positive population was selected and purified using a

FacsAria (BD Bioscience). These cells were then cultured in RPMI 1640 supplemented with 10% FCS and antibiotics. Expression of CD277 counter-receptor was followed by flow cytometry to corroborate that its surface expression was stable.

The cells obtained, were used in two different approaches to determine the identity of the CD277 counter-receptor. First, the use of cross-linking assays with Bis (sulfosuccinimidyl) suberate (BS3) a homobifunctional, water-soluble, non-cleavable and membrane impermeable crosslinker and; second, a classical co-immunoprecipitation assay from post-nuclear membranes.

In the first approach, C91T3.3 cells were incubated with 30 $\mu\text{g}/\text{mL}$ of the CD277-Fc fusion protein and then the interaction was covalently stabilized by using 3 mM of BS3. After crosslink a standard co-immunoprecipitation protocol was carried out using anti-CD277 monoclonal antibodies and protein G. A 4-12% polyacrilamide gel electrophoresis-sodium dodecyl sulfate (PAGE-SDS) and western-blot were then used to detect the CD277-Fc crosslinked, bands were then cut from the PAGE-SDS gel and sent to be identified by MALDI (Matrix-Assisted Laser Desorption/Ionization)-TOF in a mass-spectrometry assay.

The second strategy to determine the identity of CD277 counter-receptor involves a procedure to obtain the postnuclear membranes. This procedure leads us to enrich the membrane proteins and eliminate the nuclear contaminants that can interfere with the interaction between CD277 and its counter-receptor. In this approach, the C91T3.3 cells were treated with 3 mM imidazole for cell membrane lysis but not the nuclear membrane. After centrifugation, membranes were recovered and used in a classical co-immunoprecipitation assay with CD277-Fc and CTLA4-Fc as a control. A 4-12% PAGE-SDS was carried out to cut the putative counter-receptors for CD277-Fc and CTLA4-Fc, compared with a control without recombinant proteins. Bands that are in the assay but not in the controls were selected to be identified by MALDI-TOF.

2.6.2 Experimental results

The procedure to enhance the expression of CD277 counter-receptor was done twice and cells obtained were named C91T and C91T2. The expression of CD277 counter-receptor in C91T2 was higher than it was in C91T (Figure 8). A third assay to enrich the cell population expressing CD277 counter-receptor by this method was not possible, because the expression after the procedure was not stable.

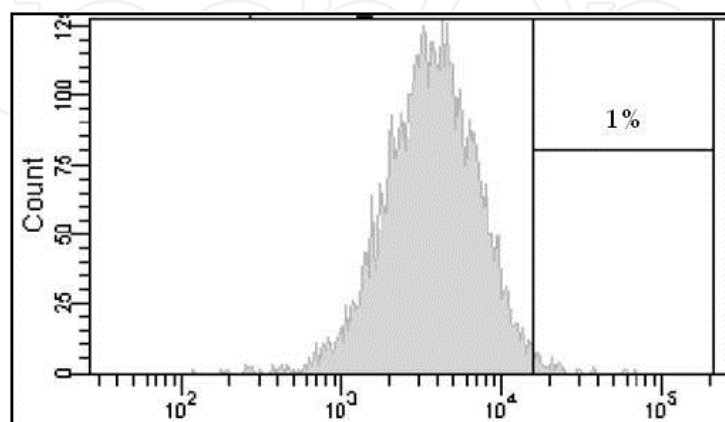


Fig. 7. Population selected from C91, C91T, or C91T2 cells. Flow cytometry was carried out to enhance the stable expression of the CD277 counter-receptor.

From C91T2 cells stained with the CD277-Fc fusion protein we proceeded to clone from the 1% most positive cells (Figure 7). Cloned cells were followed to measure the CD277 counter-receptor expression and the most positive clone obtained was selected and named C91T3.3. As shown in figure 8, increasing expression levels of CD277 counter-receptor were reached with enrichment each step. However, after C91T3.3 no more clones with stable expression of CD277 counter-receptor were obtained. Thus, the highest stable expression of CD277 counter-receptor was that obtained in C91T3.3 cells.

In the first approach to identify the CD277-counter receptor, our preliminary results shown only the homo-polymerization of CD277-Fc by the crosslinking agent BS3 and its detection by mass spectrometry (MALDI-TOF) led us to postulate that this approach is appropriate to determine the identity of CD277 counter-receptor. Nonetheless, the use of additional controls such as CTLA4-Fc is needed to further validate the results. Moreover, as can be seen in our results, it is possible that these homo-polymers mask the potential CD277-Fc - CD277 counter-receptor heterodimerization. Therefore they should be eliminated as collateral products of the reaction in order to select the appropriate band to be analyzed.

Representative 4-12% PAGE-SDS and western-blot analysis are shown in figure 9. The selected bands were analyzed by MALDI-TOF, but only the polymerization of CD277-Fc fusion protein was identified.

The use of the appropriated controls, negative and positive, is essential to validate the results. As a positive control, we used CTLA4-Fc fusion protein in C91 cells (positive by flow cytometry). However, the determination of the identity of CD277 counter-receptor is in process.

In both approaches used (crosslink and co-immunoprecipitation with post-nuclear membranes) the CD80/86 molecules were used to validate these methods. We hypothesized that if detecting CD80/86 was possible, this methodological approach would be useful for the CD277 counter-receptor identification. However, we were not able to detect CD80/86 by any of the strategies tested. Thus, even though this kind of approach has been used in similar circumstances, like the identification of the B7_H6 counter-receptor (Brandt *et al.*, 2009), technical modifications are needed in order to have the validated conditions for the detection of the CD277 counter-receptor.

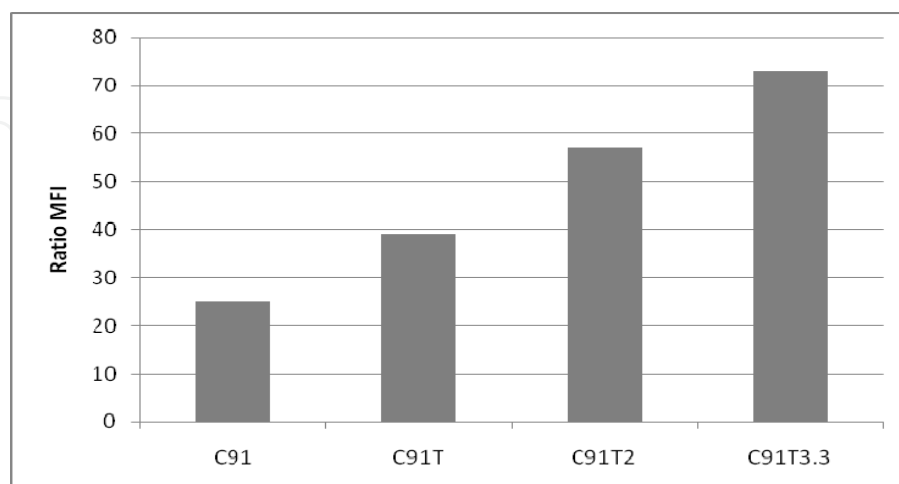


Fig. 8. Enhanced expression of CD277 counter-receptor in C91, C91T, C91T2 and C91T3.3 cells after cell sorting and cloning. Expression was followed-up using CD277-Fc fusion protein by flow cytometry.

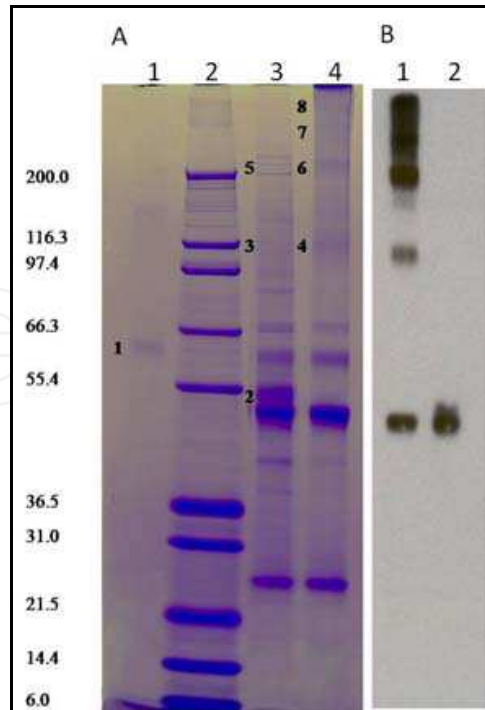


Fig. 9. A typical PAGE-SDS and western-blot analysis for the identification of CD277 counter-receptor. A) 1.- control BSA 2.- molecular weight markers, 3.- assay without BS3 and 4.- with BS3. B) Western-blot line 1 with BS3, line 2 without BS3 and line 3 molecular weight markers. Numbers indicate the selected band to be analyzed by MALDI-TOF.

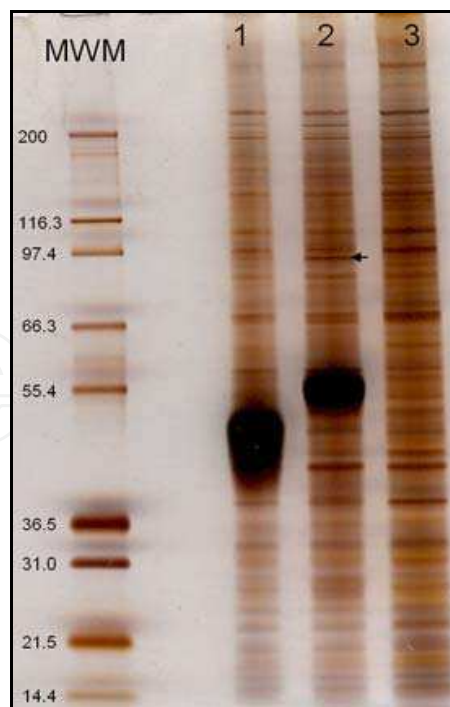


Fig. 10. A typical PAGE-SDS used in co-immunoprecipitation assays using the post-nuclear membranes. 1) Using CTLA4-Fc 2) Using CD277-Fc and 3) control without recombinant proteins. The arrow shows the band to be analyzed.

2.6.3 Monoclonal antibodies against the CD277 counter receptor

The production and use of monoclonal antibodies is another attractive strategy to enhance our knowledge of CD277 counter-receptor. However, the identity of the receptor or a recombinant counter-receptor is not available yet. Thus, we decided to obtain monoclonal antibodies to recognize this counter-receptor using cells expressing it as the immunogenic stimulus. With this rationale we immunized rats with C91T3.3 cells, which as described above, enhanced expression of the CD277 counter-receptor. Spleen cells from these immunized rats were used for the production of hybridomas following standard protocols. Important to note is the fact that when using this type of approach, the screening systems are crucial to identify the relevant hybridomas to be clone and antibodies to be purified.

Considering that the rat immune system will produce antibodies against many of the cell surface proteins of C91T3.3, a first screening was done using the CD277 counter-receptor expressing human erythroleukaemic cell line TF1. Hybridomas secreting antibodies against TF1 cells were selected for a second screening. In addition, since Raji cells stimulated with anti-CD277 mAbs show enhanced expression of the CD277 counter-receptor, these cells were used for a second screening of the clones selected in the first one. This second screening was done in parallel with an inhibitory test using C91T3.3 cells and the CD277-Fc.. With this system, hybridomas secreting antibodies against the Raji cells and with inhibitory activity on C91T3.3 were selected for cloning.

In figure 11 a representative figure is shown. The basal fluorescence of Raji cells is shown (11A). The percentage of positive non-stimulated Raji cells is shown in red and it is compared to the amount of positive stimulated Raji cells (blue) (11B). This supernatant was a potentially useful, thus it was tested for inhibitory activity on C91T3.3 cells (Figure 12). We can see that effectively the antibodies in this supernatant have an inhibitory effect on the CD277-Fc binding. Interestingly, we found that some supernatants enhance the CD277-Fc binding on C91T3.3. These clones were selected too, because they could recognize some accessory molecules for the counter-receptor.

We selected a total of 6 clones with inhibitory activity on C91T3.3 cells. We also obtained one clone whose supernatant has the ability to enhance the CD277-Fc binding on C91T3.3 cells. The identification of the proteins recognized by these antibodies is in progress.

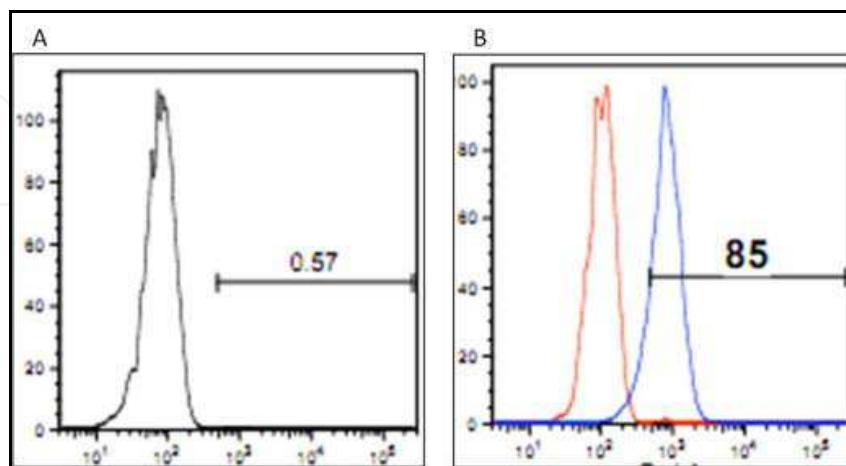


Fig. 11. Representative flow cytometry pattern of a selected clone. A) Basal fluorescence and B) fluorescence of supernatant tested on Raji non stimulated (red) and stimulated with anti-CD277 (blue). The percentage of positive cells is indicated in each pattern.

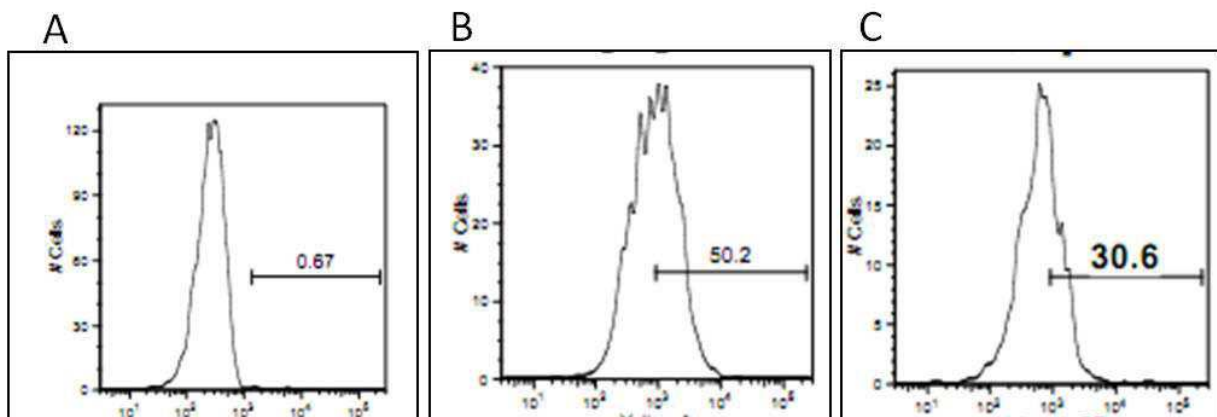


Fig. 12. Representative flow cytometry pattern of a selected clone tested on C91T3.3 cells. A) Basal fluorescence; B) Normal binding of CD277-Fc fusion protein; and C) Inhibitory test showing a reduction in the CD277-Fc binding. The percentage of positive cells is indicated in each pattern.

2.7 Perspectives on the potential use of CD277 and its counter-receptor in cancer therapy

The wide variety of function of CD277 described in this chapter shown the future potential of the use of mAbs anti-CD277 to regulate the immune response against tumor cells. The fact that stimulation of different cells by CD277 lead to the secretion of different cytokines, activation of different signalling pathways, enhance the proliferation of T cells, can be useful to modulate the immune response against tumor cells. Moreover, our results showing that Raji cells stimulated by CD277 are better killed by $\gamma\delta$ T cells are potentially useful for cancer therapy. However, it is necessary to elucidate the mechanisms by which these molecules act and to identify the counter-receptor for CD277. The use of mAbs should be a tool to elucidate the mechanisms, but to be used no to mark the tumor cells, but to modulate the response that lead to kill them.

In the near future, regulation of the expression or activity of molecules such as CD277 or its counter-receptor can be useful for enhance the anti-tumoral immune response, however it is necessary the molecular and fully functional characterization of these molecules, and the mechanisms involved in the observed functions, to be used as modulators of immune response in cancer.

3. Conclusion

Immune responses can be regulated by CD277 in different ways. CD277 affects different immune cells in various ways including inhibition of proliferation, regulation of cytokine secretion in CD4⁺ and CD8⁺ T cells, as well as activating monocytes and iDC. Moreover, CD277 promotes the expression of its counter-receptor and this effect may be important for recognition of leukaemia and solid tumor cells.

Proteomics and production and use of monoclonal antibodies are two different approaches we have tested to determine the identity of the CD277 counter-receptor.. However, although functional analysis of CD277 and its counter-receptor has demonstrated a biological effect, it is necessary to identify the counter-receptor and elucidate the signalling pathways involved in the observed response.

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The book "Advances in Cancer Therapy" is a new addition to the InTech collection of books and aims at providing scientists and clinicians with a comprehensive overview of the state of current knowledge and latest research findings in the area of cancer therapy. For this purpose research articles, clinical investigations and review papers that are thought to improve the readers' understanding of cancer therapy developments and/or to keep them up to date with the most recent advances in this field have been included in this book. With cancer being one of the most serious diseases of our times, I am confident that this book will meet the patients', physicians' and researchers' needs.

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