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Characterization of B7H6, an endogenous ligand for the NK cell activating receptor NKp30, reveals the identity of two different soluble isoforms during normal human pregnancy

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ABSTRACT

B7H6, an endogenous ligand expressed on tumor cell surfaces, triggers NKp30-mediated activation of human NK cells. In contrast, the release of soluble B7H6 has been proposed as a novel mechanism by which tumors might evade NK cell-mediated recognition. Since NK cells are critical for the maintenance of early pregnancy, it is not illogical that soluble B7H6 might also be an important factor in directing NK cell activity during normal pregnancy. Thus, this study was focused on the characterization of soluble B7H6 during the development of normal pregnancy. Serum samples were obtained from healthy pregnant women who were experiencing their second pregnancies (n = 36). Additionally, 17 of these pregnant participants were longitudinally studied for the presence of B7H6 during their second and third trimesters. Age-matched healthy non-pregnant women served as controls (n = 30). The presence of soluble B7H6 was revealed by Western blotting. A further characterization was performed using an immunoproteomic approach based on 2DE-Western blotting combined with MALDI-MS. The results show that sera from all pregnant women were characterized by the presence of two novel isoforms of B7H6, both with lower MW than the reported of 51 kDa. These isoforms were either a heavy (~37 kDa) or a light isoform (~30 kDa) and were mutually exclusive.

N-glycosylation did not completely explain the different molecular weights exhibited by the two isoforms, as was demonstrated by enzymatic deglycosylation with PNGase F. The confirmation of the identity and molecular mass of each isoform indicates that B7H6, while maintaining the C- and N-termini, is most likely released during pregnancy by a mechanism distinct from proteolytic cleavage. We found that both isoforms, but mainly the heavier B7H6, were released via exosomes; and that the lighter isoform was also released in an exosome-free manner that was not observed in the heavy isoform samples. In conclusion, we find that soluble B7H6 is constitutively expressed during pregnancy and that, moreover, the soluble B7H6 is present in two new isoforms, which are released by exosomal and exosome-free mechanisms.

1. Introduction

Members of the B7 family are important regulators of immune function in health and disease (Ceeraz et al., 2013). These molecules have been shown to be of great importance in cancer, hematologic diseases, and maternal-fetal tolerance (Greaves and Gribben, 2013;

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Petroff and Perchellet, 2010).

B7H6 (NCR3LG1), a tumor-specific member of the B7 family, is a type I 51 kDa protein with 454 amino acids, which was first reported to be largely expressed on various types of human tumors (Brandt et al., 2009). B7H6 is a highly glycosylated protein with seven putative N-glycosylation sites, which assist in the maintenance of its conformation and interaction with its cognate receptor on NK cells (Li et al., 2011; Xu et al., 2015). Building on reports that show B7H6 is up-regulated in pro-inflammatory environments such as sepsis, liver damage, auto-immunity, and cancer (Zou et al., 2015; Salimi et al., 2016; Rusakiewicz et al., 2013; Matta et al., 2013), several studies have focused on the use of B7H6 as a marker of tumor progression and a potential therapeutic target (Cao et al., 2015; Chen et al., 2014; Zhang et al., 2014; Peipp et al., 2015; Wu et al., 2015a; Kellner et al., 2012; Zhou et al., 2015; Wu et al., 2015b; Semeraro et al., 2015; Pesce et al., 2015). Little is known, however, about the factors that regulate the expression of B7H6, although this molecule has shown histone deacetylase (HDAC)-dependent expression (Fiegler et al., 2013).

Recently, it has been shown that B7H6 is indeed a functional ligand for the NK cell-activating receptor NKp30, which has the capacity to recognize not only cell surface B7H6 (Brandt et al., 2009), but also the endogenous ligand BAG6 (Pogge Von Strandmann et al., 2007), promoting the elimination of tumor cells or dendritic cell maturation, respectively. Similar to other cellular ligands of activating NK receptors (for instance MICA/B and ULBPs), which can be released from tumor cells (Chitadze et al., 2013), B7H6 might also be shed from the cell surface to the extracellular space, which would represent an important regulatory mechanism with respect to NK cells. The release of a soluble form of B7H6 has been shown to impact not only NKp30 expression, but also NK cell activity in patients with different tumors (Semeraro et al., 2015; Pesce et al., 2015). Interestingly, while the release of soluble ligands is a mechanism notably exploited by tumors, it may also be observed during pregnancy. For example, the presence of soluble NKG2D ligands has been reported in the serum of pregnant women, whereby these bioactive ligands are able to regulate the expression of their activating receptor NKG2D on peripheral blood mononuclear cells, as a novel mechanism of maternal-fetal immunoprotection (Mincheva-Nilsson et al., 2006; Hedlund et al., 2009).

With respect to activation and tumor regulation, B7H6 apparently displays behavior similar to the NKG2D/NKG2D ligand axis, but the presence of this molecule in pregnancy has yet to be reported; for this reason we have focused the present study on the detection of soluble B7H6 in the serum of women during the first, second, and third trimesters of normal pregnancy. We demonstrate, for the first time, the presence of soluble B7H6, starting at early pregnancy, as well as two unique isoforms of B7H6, differing in weight and post-translational modifications, that are possibly processed by as of yet unknown mechanisms.

2. Materials and methods

2.1. Recruitment of pregnant participants

Recruitment of the pregnant participants took place at the Hospital Materno Infantil “Esperanza López Mateos” (Secretaría de Salud, Jalisco, México). Thirty-six pregnant women between 19 and 40 years old, who were experiencing their second pregnancies, were recruited for the study. Women with preeclampsia, preexisting diabetes, and other clinical or gynecological/obstetric abnormalities were excluded from the study. All pregnant women were screened for gestational diabetes mellitus at 28 weeks of gestation, and all were negative. Only women with healthy full term pregnancies were selected for final inclusion in the study. After completing the selection criteria for recruitment, serum samples were obtained from the 36 pregnant participants during their first trimester of pregnancy (range of 4–12 weeks). Additionally, 17 of these pregnant participants were also asked to provide serum samples during their second (13–23 weeks) and third trimesters (26–38 weeks) of pregnancy. Thirty age-matched healthy non-pregnant women were selected from the general population and served as controls (with an age range of 18–36).

Serum samples were maintained at −80 °C until use.

2.2. Ethics

Before initiation, the research protocol was submitted to the relevant Institutional Review Board Committees (Comisiones de Investigación, Ética y Bioseguridad del Centro Universitario de Ciencias de la Salud, Universidad de Guadalajara) and was approved and classified as a study without undue risks or burdens that complied with the institutional requirements ensuring appropriate ethical and biosecurity conduct. Moreover, the study was considered to be in accordance with the guidelines of the Official Mexican Standard (Norma Oficial Mexicana) for research in humans, as well as the guidelines of the Helsinki Declaration. After an explanation of the study’s aims and data usage, a letter of informed consent was voluntarily signed by the women in the pregnant and control groups prior to enrollment in the study.

2.3. Western blotting analysis

Each sample was directly mixed with SDS-PAGE sample loading buffer (300 mM Tris-HCl, pH 6.8, SDS 20%, 100 mM DTT, bromophenol blue 0.001%, glycerol 20%). The samples were loaded onto a 10% polyacrylamide gel and were run at 140 V/1:30 h. For immunoblot analyses, the serum proteins were transferred onto a PVDF membrane (Bio-Rad) using the Mini Trans-Blot® Cell (Bio-Rad). Samples were transferred overnight at 40 V and blocked with TBS-Tween-20 0.1% with 5% blotting-grade blocker non-fat dry milk (Bio-Rad) for 3 h at room temperature. After the blocking step, the membranes were incubated overnight at 4 °C with rabbit anti-B7H6 polyclonal antibody (ab138330, Abcam) diluted in TBS-Tween-20 0.1% with 0.5% blotting-grade blocker (Bio-Rad). Alternatively, this antibody plus one additional rabbit anti-B7H6 C-term polyclonal antibody (ab138588, Abcam) were also used as an internal control of the conventional 51 kDa B7H6 in whole cell protein extracts prepared from various cervical cancer cell lines (HeLa, SiHa, and C33A). Mouse anti-human transferrin monoclonal antibody (MAB5746, R & D Systems) was also used as loading control for proper interpretation of the Western blot assays. The membranes were next washed three times for 10 min each with TBS-Tween-20 0.1% and incubated with the respective goat IgG-HRP secondary antibody (sc-2004 and sc-2005, Santa Cruz Biotechnology, Inc) diluted in TBS-Tween-20 0.1% with 0.5% blotting-grade blocker non-fat dry milk (Bio-Rad) for 1 h at 37 °C. The membranes were next washed three times for 10 min each with TBS-Tween-20 0.1%, giving a final wash with straight TBS, and then visualized using the MicroChemi 4.2 imaging system (DNR Bio-Imaging Systems).

2.4. Two-dimensional electrophoresis of B7H6

For two-dimensional electrophoresis (2DE), ReadyStrip™ IPG Strips (linear pH 3–6, 7 cm; Bio-Rad) were used. These gels were rehydrated overnight with 12 µg of serum protein in 125 µL of rehydration buffer (7 M urea, 2 M thiourea, CHAPS), 1 M DTT, and 20 mM IPG Buffer (3–10). The first-dimensional separation was performed using the Ettan IPGphor™ 3 Isoelectric Focusing (IEF) Unit (GE Healthcare Life Sciences) with four steps: 300 V/2:30 h; gradient 1000 V/0:30 h; gradient 5000 V/1:30 h, and 5000 V/0:35 h. After completion of the IEF, the ReadyStrip™ IPG Strips containing the focused proteins were equilibrated by the next two incubation steps: with DTT 1% w/v 20 min, and subsequently with iodoacetamide 2.5% w/v for 20 min. The ReadyStrip™ IPG Strips were then transferred onto 10% polyacrylamide slab gels and the two-dimensional separation was carried out utilizing

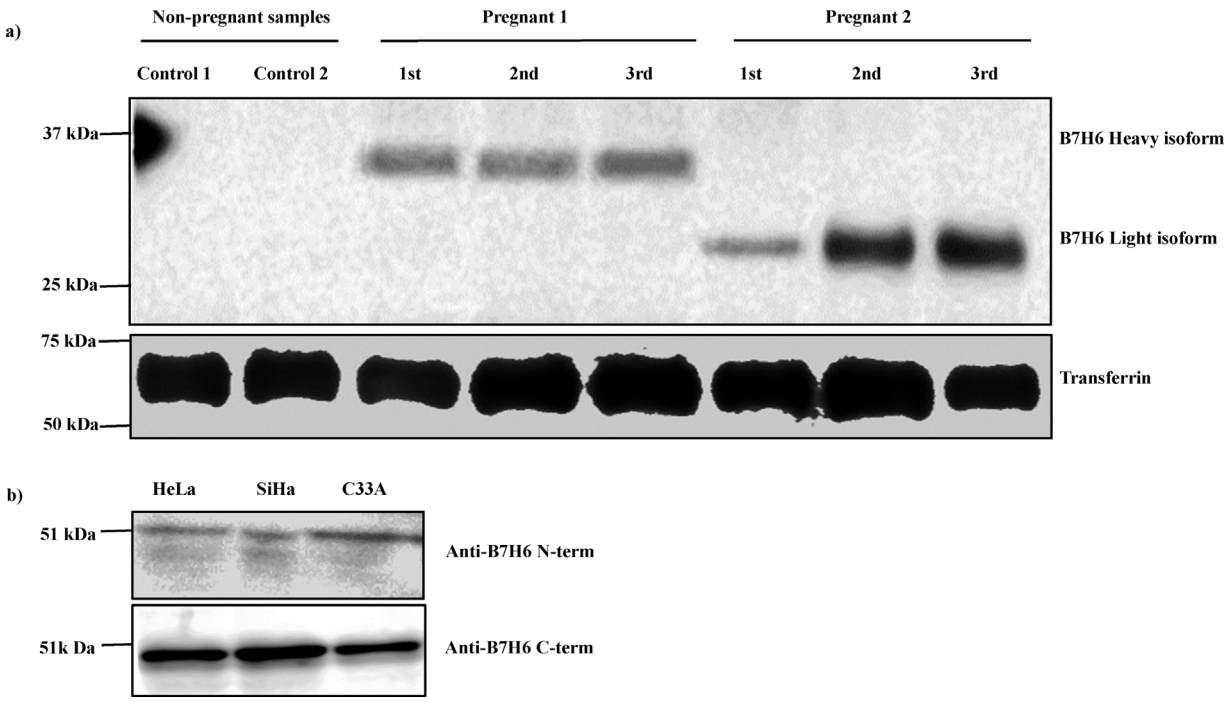

Fig. 1. Two new isoforms of B7H6 are found and maintained during normal pregnancy. Sera from a total of 36 healthy pregnant women were analyzed by Western blot for the determination of soluble B7H6. Age-matched healthy non-pregnant women served as controls (n = 30). Sera from all pregnant women were characterized by the presence of two new isoforms of B7H6. These mutually exclusive isoforms were either a heavy (~37 kDa) or a light isoform (~30 kDa) and were maintained throughout pregnancy. (A) Representative examples of the maintenance of both B7H6 isoforms during the three trimesters of pregnancy compared with samples from healthy women controls. Transferrin was used as a loading control. (B) Internal control for the detection of the conventional 51 kDa B7H6 protein, which is present in whole cell extracts from cervical cancer cell lines (HeLa, SiHa, and C33A); an additional anti-B7H6 C-terminal antibody was also tested. First trimester (1st), second trimester (2nd) and third trimester (3rd) of pregnancy.

the Mini-PROTEAN® Tetra System (Bio-Rad). Proteins were visualized by Coomassie Blue (Bio-Rad).

2.5. In-gel digestion for mass spectrometric characterization of B7H6

Two-dimensional gels (2DE) were stained with Coomassie Blue (Bio-Rad) and Western blot-positive spots were excised from the gels and analyzed. Briefly, the excised gel spots were mixed with 0.01 M DTT-0.1 M Tris, pH 8.5 and the tubes were placed in a heating block at 55 °C for 1–2 h and then cooled at room temperature; the solution was removed and replaced with 0.015 M iodoacetamide-0.1 M Tris, pH 8.5. Then, the solution was removed and the gel pieces were washed twice with 30% acetonitrile-0.05 M Tris, pH 8.5 for 15 min with shaking. The gel pieces were then dehydrated by soaking for a few min in acetonitrile. The acetonitrile was removed and the gel pieces were completely dried for 30 min in a Vacuum concentrator (Eppendorf). After rehydratation of the gels, peptides were extracted twice using 50% acetonitrile-2% TFA, and the combined extracts were dried and then resuspended in matrix solution. Matrix-assisted laser desorption/ionization mass spectrometry (MALDI-MS) analysis was performed on the digests using a PerSeptive Voyager DE-Pro mass spectrometer in linear mode. For the peptide mass search, the average peptide masses were entered into the following search programs to search NCBI and/or GenPept databases for protein matches: ProFound available at http://129.85.19.192/profound-bin/WebProFound.exe, and MS-Fit available at http://prospector.ucsf.edu.

2.6. Analysis of B7H6 glycosylation by enzymatic removal of N-glycans

The deglycosylation reaction was carried out with peptide-N-glycosidase F, commonly referred to as PNGase F (P7367, Sigma-Aldrich), according to the manufacturer's instructions. This assay was performed on 4 different serum samples: two positive samples for the B7H6 heavy isoform and two for the B7H6 light isoform. Briefly, samples were mixed with the reaction buffer and were incubated at 80 °C/10 min; next, 5 units of PNGase F per tube were added and the samples were incubated at 37 °C for 3 h. The mixtures were resolved and analyzed by SDS-PAGE and immunoblot analysis, as already described above. Additionally, the highly N-glycosylated protein MICA/B was used as reference to confirm the efficiency of PNGase F (anti-MICA/B, sc-137242, Santa Cruz Biotechnology, Inc).

2.7. Identification of B7H6 in purified serum exosomes

Exosome isolation was performed with the Total Exosome Isolation Reagent (Cat. 4478360, Invitrogen™) according to the manufacturer's instructions. Briefly, five samples positive for the B7H6 heavy isoform and five samples positive for the B7H6 light isoform were pooled after being thawed in a water bath at room temperature until the samples were completely liquid and then centrifuged at 2000*g*/30 min to remove any cellular debris. The isolation reagent (100 µL) was then added to the debris-free sera, which was subsequently vortexed and then incubated for 30 min; the samples were next centrifuged at 12,000*g*/10 min, resulting into two phases, the first one being the exosome-free supernatant and the second one the exosome pellet. The samples were resolved and analyzed by SDS-PAGE and immunoblotting assays as already mentioned. In parallel, a classical marker of vesicles with features of exosomes (anti-CD63 cat. 353013 BioLegend) was used to confirm the presence of B7H6 isoforms in exosome-enriched fractions.

3. Results

3.1. Detection of B7H6 in serum during development of normal pregnancy

Accumulating evidence supports the notion that NK cells play a critical role in the maintenance of pregnancy through several mechanisms; for instance, secretion of soluble NKG2D ligands from

placental explants into the circulation might facilitate the maternal immune tolerance through NKG2D down-regulation on peripheral blood lymphocytes (Hedlund et al., 2009). NKp30, another key NK cell activating receptor, has begun to gain attention in maternal tolerance; however, the nature of its ligands during development still remains unknown and poorly studied. Hence, we moved our effort in the identification of B7H6 during normal human pregnancy. Thirty-six pregnant women, who were experiencing their second pregnancies, were recruited for the study. In parallel, thirty age-matched healthy non-pregnant women served as controls. Circulating B7H6 was analyzed in each serum sample using the Western blot technique. The results interestingly showed that one of either of two different bands, both of lower molecular weight with respect to the 51 kDa reported for B7H6 (Brandt et al., 2009), was observed in all pregnant women; in contrast, all sera from the control group were negative, as seen in representative samples depicted in Fig. 1a. Remarkably, of the total 36 pregnant sera analyzed during the first trimester, 28 (78%) demonstrated the presence of a protein band of approximately 37 kDa and the remaining 8 (22%) demonstrated the presence of an ~30 kDa band. Each serum sample was either positive for the ~37 kDa band or the ~30 kDa band, never both (data not shown). Additionally, from the 36 pregnant women, 17 were longitudinally studied for the presence of B7H6 during their second and third trimesters and interestingly, B7H6 was maintained throughout pregnancy with an approximately 3:1 heavy to light isoform prevalence (data not shown). Representative examples of the maintenance of both B7H6 isoforms during the three trimesters of pregnancy are depicted in Fig. 1a. For better interpretation of the Western blot assays, transferrin was assessed as loading control for serum samples. In the same Fig. 1a, it can be appreciated that the loading control bands show uniformity in each lane. Due to the fact that only the 51 kDa form has conventionally been described, we also examined the anti-B7H6 N-term polyclonal antibody plus one additional anti-B7H6 C-term polyclonal antibody in whole cell protein extracts prepared from various cervical cancer cell lines (HeLa, SiHa, and C33A); as expected, both antibodies identified only the 51 kDa form of B7H6 (Fig. 1b).

3.2. Confirmation of the two isoforms of B7H6 using 2DE and mass spectrometry

Confirmation of the heavy and light isoforms of B7H6 was performed in at least four different samples. Fig. 2 shows two representative examples, one positive for each band, which were used for the 2DE and mass spectrometry studies. Each of the serum samples was first run on IPG strips with range pH 3–6 and then on 10% polyacrylamide gel. The reactive spots (arrows) observed on the PVDF membrane were identified in the gel replicates. The corresponding spots were manually cut out with new sterile micropipette ends, collected in sterile Eppendorf tubes, and digested with trypsin. Tryptic peptides from spots were analyzed by MALDI-Time of flight (TOF) spectrum and were compared with calculated values. The spots marked with an arrow were identified as B7H6 with Swiss Prot access number 320461732 (Table 1). These results confirmed the presence of two new isoforms of B7H6, a heavy isoform and a light isoform, both present during the three trimesters of pregnancy. Additionally, both spots identified as B7H6 had an isoelectric point that ranged from 4 to 5.

3.3. Enzymatic N-glycan analysis of the heavy and light B7H6 isoforms

Due to the fact that B7H6 is a highly glycosylated member of the B7 family, we wanted to further investigate and learn if the dissimilar molecular weights between the two isoforms could be attributed in part to post-translational glycosylation. Thus, an enzymatic digestion with PNGase F was performed in at least four independent experiments. Fig. 3 shows two representative serum samples containing the heavy isoform (~37 kDa) and the light isoform (~30 kDa). The treated heavy isoform sample did not show any change in molecular weight; in contrast, the sample with the light B7H6 isoform showed a clear decrease in molecular weight as compared to the untreated control. These results showed that the heavy isoform might not contain significant amounts of N-glycans; this is in contrast to the light isoform, which was affected by PNGase F. As currently there have not been any data reported with respect to N-glycosylation sites of the ~37 and ~30 kDa B7H6 isoforms, we therefore designed a conformational experiment, in which the whole cell lysate from HeLa cells, which express the conventional 51 kDa B7H6 protein, was treated with PNGase F. The enzymatic removal revealed the presence of lighter bands on the blot; thus, we could confirm the efficiency of the PNGase F on the conventional B7H6 isoform (data not shown). Moreover, using a N-glycosylated protein as reference ratified the efficiency of the enzyme. Taking advantage of the fact that MICA and MICB (which are also NK cell activating ligands) are highly glycosylated proteins, we used MICA/B for the same purpose. MICA is a protein that has been proven to be susceptible to PNGase F activity (Mellergaard et al., 2014). After enzymatic treatment with PNGase F, the molecular weight of MICA/B substantially diminished as shown in Fig. 3. With this result, we were able to reconfirm the competence of the PNGase F on N-glycosylated proteins.

3.4. Exosome-dependent and -independent mechanisms are involved in the release of B7H6 isoforms

The liberation of NK-activating ligands to the extracellular space, through exosomal release for instance, is a mechanism of regulation highly exploited during pregnancy in order to favor the maintenance of maternal/fetal tolerance. For this reason, we examined whether B7H6 is liberated to the serum in an exosome-dependent or -independent manner. Pooled-samples that presented either the ~37 kDa (five samples) or ~30 kDa (five samples) isoforms were processed using a commercial exosome isolation kit. In the pool of samples containing the light isoform, B7H6 appears to be observed almost equally in both fractions, albeit marginally more prominently in the exosome-free fraction; in contrast, the pool of samples with the heavy isoform demonstrated staining principally in the exosomal fraction of the samples, and very weakly in the exosome-free fraction, thus suggesting that the unique characteristics of each of the isoforms favors their respective routes of liberation (Fig. 4). Additionally, a control for exosome enrichment was made in order to be certain of the location of B7H6 in the exosome fraction. Fig. 4 shows that a classical marker of vesicles with features of exosomes (CD63) was strongly confined to the exosome fraction. As expected, this staining was also observed in the unfractionated control.

4. Discussion

A challenging question with respect to pregnancy is that of why the fetal-placental unit is not rejected by the maternal immune system. Several mechanisms have been proposed in order to explain this tolerance; however, to date, this remains a subject of intense debate.

NK cells are undoubtedly important during pregnancy (Colucci and Kieckbusch, 2015); these cells have been highly studied due to their potent cytotoxic action against virus-infected cells and tumors (Vivier et al., 2008; Vivier et al., 2012). During pregnancy, however, these cells also mediate a contrasting regulatory or protective mechanism through the secretion of soluble mediators such as cytokines, chemokines, and growth factors that contribute to the migration of the trophoblast and the reorganization of the uterine niche for the development of the embryo (Hanna et al., 2006; Fraser et al., 2015).

The activation of NK cells is controlled by a delicate balance between triggering and inhibitory receptors (Vivier et al., 2012). Among the most common types of activating receptors are: NKG2D, NKp46, and NKp30 (Brusilovsky et al., 2012). Due to its ability to control the secretion of pro-angiogenic factors and cytotoxicity, NKp30 on decidual

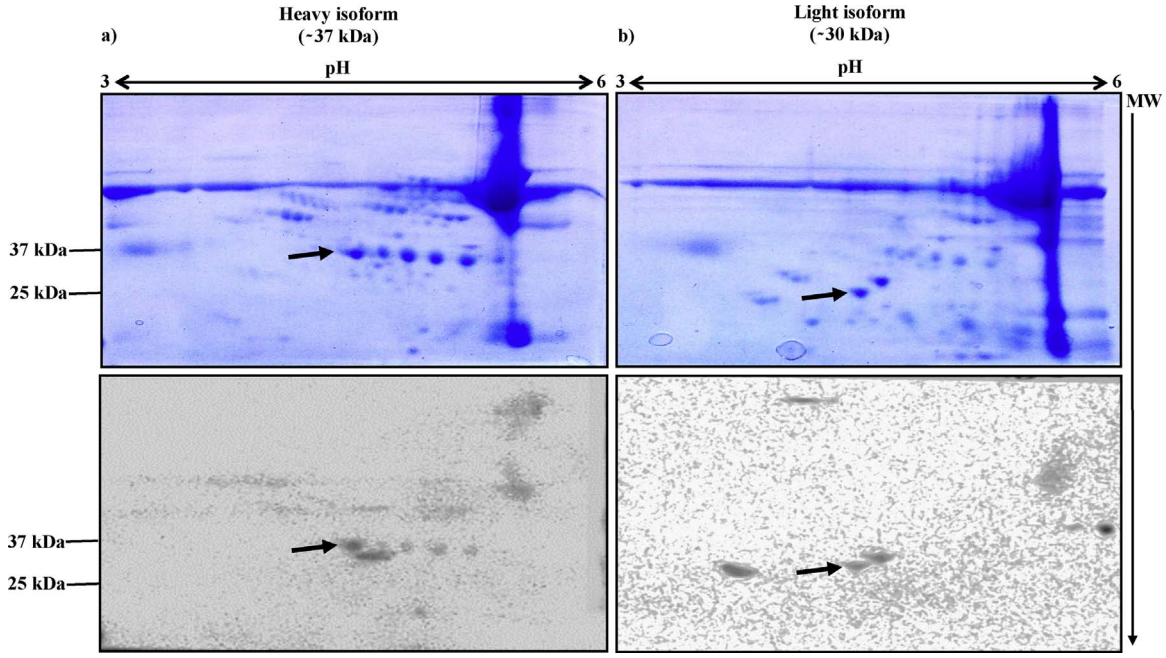

Fig. 2. Two-dimensional gel electrophoresis analysis of the heavy (~37 kDa) and light (~30 kDa) B7H6 isoforms. Each of the serum samples was first run on IPG strips with range pH 3–6 and then on 10% polyacrylamide gel. The reactive spots (arrows) observed on the PVDF membrane were identified in the gel replicates. Representative examples of the ~37 kDa heavy isoform or the ~30 kDa light isoform are shown in (A) and (B), respectively. Both spots identified as B7H6 had an isoelectric point between 4 and 5. MW: molecular weight.

NK cells plays an important role during pregnancy; in addition, there are reports that support that the up-regulation of some NKp30 isoforms is associated with spontaneous abortions (Hanna et al., 2006; El Costa et al., 2008; Shemesh et al., 2015). It remains to be seen whether decreased NKp30 levels or different types of NKp30 signaling (B7H6 isoforms) correlate with fetus health. Even though NKp30 is a fundamental receptor, as of yet only few activating ligands have been described; one such is B7H6, the most recently described membrane ligand that corresponds to the B7 family (Brandt et al., 2009); additionally, there are some reports that indicate that B7H6 may also be present in a soluble form (Matta et al., 2013; Schlecker et al., 2014). B7H6 is considered as a stress ligand and, similar to MICA/B, is overexpressed in tumors. However, in contrast to MICA/B, a role for B7H6 during pregnancy has not yet been described.

Here, for the first time, we have reported the presence of the soluble B7H6 immunoligand in the serum of pregnant women during the development of pregnancy. Interestingly, B7H6 was restricted to two different presentations of molecular weights: one heavy isoform of ~37 kDa, and a light isoform of ~30 kDa, both of which were mutually exclusive (Fig. 1a). Both of these isoforms were found to be maintained during the three trimesters of pregnancy, with the heavier isoform predominating by about 3:1. No association was found between the different trimesters and the presence of heavy or light isoforms. Therefore, based on these findings, we can speculate that NK cells play an important role throughout the entire pregnancy, despite the fact that it has been generally recognized that these cells act predominantly during early pregnancy and then decrease until birth of the newborn (Bartmann et al., 2014). The presence of soluble B7H6 may impact the function of peripheral NK cells via the NKp30 receptor. This has been observed in patients with ovarian carcinoma and neuroblastoma; interestingly, these patients showed that the concentration of soluble B7H6 significantly correlated with the down-regulation of NKp30, consequently affecting NK cell functions, including IFN-γ production (Semeraro et al., 2015; Pesce et al., 2015). Thus, the regulation of NKp30 through its soluble ligand B7H6 might have a physiological impact on circulating peripheral blood NK cells during pregnancy.

Similar to other ligands that are recognized by NK cells, such as MICA/B, B7H6 is also a molecule that is highly glycosylated (Li et al., 2011); this helps this ligand to maintain its proper structural form (Xu et al., 2015). Thus, we wondered if the difference between the molecular weights of the two isoforms might be due to post-translational glycosylation. We found that the differences between molecular weights could not be completely attributed to N-glycosylation, due to the fact that PNGase F treatment did not modify the molecular weight

Table 1
Tryptic peptides from spots marked by arrow measured by MALDI-TOF.

B7H6	Measured mass, amu (average)	Calculated mass, amu (average)	Error, amu	Sequence position		Sequence
				Start	End	
Light isoform[a]	1337.2764	1337.5031	316.3	375	384	R.DNPDLCQCCRI
	1993.4456	1993.0641	114.8	184	201	K.FPHPIEISEDVITGPTIKN
	1993.4456	1993.0726	114.8	232	249	R.HASLHTPLRSNFTLTAARH
	2810.5742	2810.2800	462.0	161	183	K.YMCESSGFYPEAINITWEKQTQKF
Heavy isoform[b]	930.2688	930.4527	−198	250	257	R.HSLSETEKT
	2419.6812	2419.2562	176	385	407	R.IDPALLTVTSGKSIDDNSTKSEKQ
	2626.6091	2626.4233	70.8	131	155	K.AQGTVQLEVVASPASRLLLDQVGMKE
	2810.6711	2810.2800	139	161	183	K.YMCESSGFYPEAINITWEKQTQKF

Measured masses from the MALDI-TOF spectrum were compared to calculated values. Masses listed represent [a]17.4% and [b]15.2% sequence coverage.

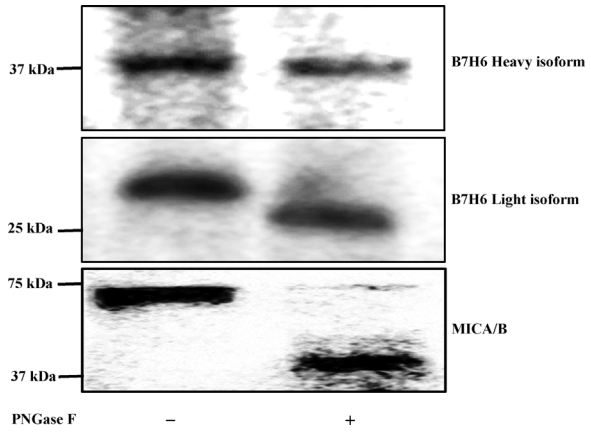

Fig. 3. Enzymatic N-glycan analysis of the heavy and light B7H6 isoforms. Deglycosylation reaction was performed with PNGase F. This assay was performed in 4 different serum samples: two positive samples for the B7H6 heavy isoform and two for the light isoform. The mixtures were resolved and analyzed by SDS-PAGE and immunoblot analysis. The treated heavy isoform sample did not show any change in molecular weight; in contrast, the sample with the light B7H6 isoform showed a decrease in molecular weight as compared to the untreated control (upper and middle blots). A highly N-glycosylated protein (MICA/B) was used as reference to confirm the efficiency of PNGase F (bottom blot).

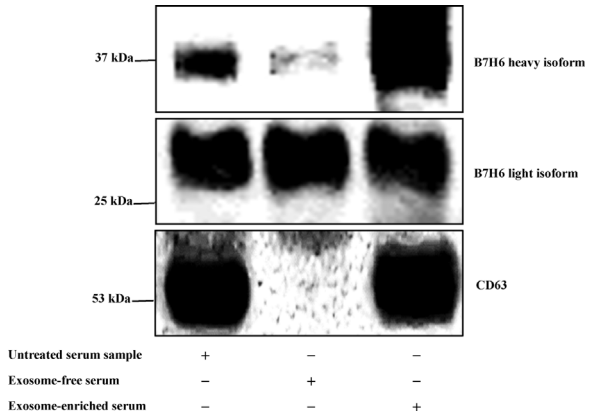

Fig. 4. B7H6 is released by exosomal and exosome-free mechanisms. Pooled-samples that presented either the ~37 kDa (five samples) or ~30 kDa (five samples) isoforms were processed using a commercial exosome isolation kit. Forty μg of total isolated protein per sample were then resolved and analyzed by SDS-PAGE and immunoblotting assays. Upper blot: the pool of samples with the heavy isoform demonstrated staining principally in the exosomal fraction of the samples, and very weakly in the exosome-free fraction. Middle blot: in the pool of samples containing the light isoform, the staining was almost equally in both fractions, albeit marginally more prominently in the exosome-free fraction. Bottom blot: a control marker for the exosome fraction (CD63) was used in order to be certain of the identification of exosomes. It is important to also note that 40 μg of serum proteins were run as an unfractionated control in all blots.

with respect to the heaviest isoform, as shown in Fig. 3. Interestingly, a distinct effect was observed in the light isoform, which demonstrated a decrease in the molecular weight, due to the loss of at least one N-glycosylation residue. However, we cannot discard the possibility that other post-translational modifications might also explain the dissimilar molecular weights; one such might be palmitoylation, a mechanism that has been previously described in the case of MICA, which facilitates the shedding of this ligand from the cell membrane (Aguera-Gonzalez et al., 2011). Although there are as of yet no reports discussing whether B7H6 is post-transcriptionally palmitoylated or not, in silico prediction of the B7H6 sequence has shown possible sites of palmitoylation, which might then explain the difference between the molecular weights presented by the two isoforms.

Different release mechanisms for soluble proteins have been reported. For example: via proteolytic cleavage, micro-vesicle release (100 nm–1 μm), apoptotic bodies (50–500 nm) or exosomes (30–100 nm). The liberation of exosomes has been described as an important release mechanism during pregnancy (Urbanelli et al., 2013) and has been reported to increase during the progression to later stages (Mitchell et al., 2015). Exosomes released during pregnancy are capable of regulating the immune response due to their ability to regulate NK cells, T lymphocytes and monocytes (Mincheva-Nilsson et al., 2006; Atay et al., 2011; Stenqvist et al., 2013). In the particular case of B7H6, it was initially thought that this ligand was only released via exosomes in patients with sepsis (Matta et al., 2013). However, it has more recently been reported that B7H6 may be released by proteolytic cleavage in tumor cells (Schlecker et al., 2014). Thus, one of our goals was to determine if B7H6 was confined mostly to the exosome-rich or the exosome-free fraction. Aside from the fact that we observed that both the light and heavy isoforms were present in the exosome-free portion of the serum, more notable was the fact that the heavy isoform was found mainly in the exosome-rich portion. Based on these results, we can speculate that the heavy isoform might be found anchored in the exosome membrane, while the light isoform might not contain the same trans-membrane portion, thus facilitating its release to the extracellular environment (Fig. 4). These two findings are similar to those reported by Fernandez-Messina et al. (Fernandez-Messina et al., 2010), wherein ULBP3 was observed to be released primarily via an exosomal pathway, while ULBP2 was released via proteolytic cleavage. Also, Ashiru et al. (Ashiru et al., 2010) demonstrated that MICA *008 is released via the exosomal pathway, while the structurally similar MICA *019 is released via proteolytic cleavage. Continuing with this idea leads to the supposition that the release of B7H6 via one pathway *versus* another might confer different functions to the peripheral blood NK cells during the progression of the pregnancy.

The challenge ahead will be to elucidate the source of B7H6, keeping in mind always that the placenta is an important producer of immunoregulatory mediators. Additionally, it will be important to understand the structural differences between the two isoforms, and subsequently the biological consequences of these differences, in order to better clarify the role of the NKp30/B7H6 axis during the development of pregnancy.

5. Conclusions

Our data provide the first evidence that soluble B7H6 is constitutively expressed starting at the early stages of normal pregnancy. Moreover, our study showed the presence of two new isoforms characterized by different molecular weights, both of which were liberated by exosomal and exosome-free mechanisms, with the heavier isoform mostly confined to the exosome-rich portion.

Conflict of interest statement

The authors individually declare that there were no financial or commercial conflicts of interest.

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