Induction of B7-H6, a ligand for the Natural Killer cell activating receptor NKp30, in inflammatory conditions

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Short title: B7-H6 in inflammatory conditions

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Key points

- **B7-H6** transcripts, B7-H6 cell surface expression and sB7-H6 can be induced in inflammatory conditions *in vitro* and *in vivo*
- B7-H6 is expressed on pro-inflammatory CD14⁺CD16⁺ monocytes in sepsis conditions and is linked to an increased mortality

Abstract

B7-H6, a member of the B7 family of immunoreceptors, is a cell surface ligand for the NKp30 activating receptor expressed on Natural Killer (NK) cells. B7-H6 is not detected in normal human tissues at steady-state but is expressed on tumor cells. However, whether B7-H6 can be expressed in other conditions remains unknown. We analyzed here the pathways that lead to the expression of B7-H6 in non-transformed cells. *In vitro*, B7-H6 was induced at the surface of CD14⁺CD16⁺ pro-inflammatory monocytes and neutrophils upon stimulation by ligands of Toll-like receptors or pro-inflammatory cytokines such as interleukin-1β (IL-1β) and tumor necrosis factor α (TNFα). In these conditions, a soluble form of B7-H6 (sB7-H6) was also produced by activated monocytes and neutrophils. *In vivo*, B7-H6 was expressed on circulating pro-inflammatory CD14⁺CD16⁺ monocytes in a group of patients in sepsis conditions, and was linked to an increased mortality. sB7-H6 was selectively detected in the sera of patients with Gram⁻ sepsis and was associated with membrane vesicles that co-sedimented with the exosomal fraction. These findings reveal that B7-H6 is not only implicated in tumor immunosurveillance but also participates to the inflammatory response in infectious conditions.

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Key words:
Natural killer Cells, NKp30, B7-H6, pro-inflammatory monocytes, inflammation, sepsis
**Introduction**

Natural Killer (NK) cells are cytolytic and cytokine-producing lymphocytes that can recognize a variety of cells in distress.\(^1\) Cells undergoing microbial infection, tumor transformation as well as chemical or physical insults become NK cell targets when they express stress-induced surface molecules that interact with activating NK cell surface receptors. The recognition of NKG2D ligands expressed by target cells via NKG2D expressed on NK cells represents a prototypical illustration of this stress-induced self mode of innate immune recognition.\(^2\)

NK cells express at their surface a group of activating receptors that are Ig-like superfamily members and are referred as to Natural Cytotoxicity Receptors (NCRs). NCRs include NKp46 (NCR1, CD335), NKp44 (NCR2, CD336) and NKp30 (NCR3, CD337).\(^3\) Although these molecules have no homology, they have been grouped as NCRs based on the similarities in their expression profile, their oligomeric structure and their function.\(^4\) The characterization of the NCR ligands is still incomplete. While many data support a central role of NCR in tumor surveillance,\(^5\)-\(^8\) the first NCR ligands to be identified were viral structures, in particular the influenza haemaglutinin for NKp46\(^9\) and the human cytomegalovirus pp65 tegument protein for NKp30.\(^10\) Later the HLA-B associated transcript 3 (BAT3) protein was shown to bind and trigger NKp30.\(^11\) This nuclear protein now referred as to BAG6 (http://www.genenames.org/data/hgnc_data.php?hgnc_id=13919) is ectopically found at the plasma membrane upon stress but absent from tumor cells susceptible to NK cell lysis. We identified B7-H6 (NCR3LG1, http://www.genenames.org/data/hgnc_data.php?hgnc_id=42400) as a ligand of NKp30.\(^12\)-\(^13\) B7-H6 transcripts have not been detected in normal adult tissues. However B7-H6 is present on a broad panel of hematopoietic and non-hematopoietic tumor cells including lymphoma, leukemia, melanoma, and carcinoma as well as on primary tumor blood cells.\(^12\) The pattern of B7-H6 expression, which appears so far to be limited to tumor cells, is thus another example of stress-induced self recognition by NK cells. In the case of NKG2D ligands, several mechanisms have been shown to regulate their expression. For example, genotoxic stress
was shown to up-regulate NKG2D ligands on cell lines, whereas interfering with the DNA damage pathway in tumor cells inhibited their constitutive expression. In contrast to NKG2D ligands, the regulation of B7-H6 expression is unknown. We studied here the mechanisms that govern the induction of B7-H6 on primary cells as well as its consequences in vitro and in vivo.
Methods

A detailed description of all experimental methods can be found in the supplemental Methods on the Blood website.

Cells and reagents

PBMCs were isolated from healthy volunteer donors using Ficoll-Paque Plus (GE Healthcare) density centrifugation. Human monocytes were purified with CD14 microbeads (Miltenyi) according to the manufacturer’s instructions. Cell purity was 90–98%. Human neutrophils were isolated from peripheral blood of healthy donors using a dextran-Ficoll method. Human NK cells were purified with a human NK cell isolation II Kit (Miltenyi) according to the manufacturer’s instructions. Cells were cultured in complete RPMI 1640 medium (GIBCO) supplemented with 100 U/mL penicillin/streptomycin (GIBCO), 1 mM sodium pyruvate (GIBCO), 10% heat-inactivated FBS (LONZA). Human PBMCs (3 x 10^6 cells), neutrophils (2 x 10^6 cells) or monocytes (5 x 10^5 cells) were seeded into 24-well plates (BD). Cells were then incubated at 37°C with 5% CO₂ in the presence or absence of the following stimuli: 300 ng/ml synthetic bacterial lipoprotein (Pam3CSK4), 10 µg/ml zymosan, 10^8 cells/ml heat-killed A. laidlawii (HKAL), L. monocytogenes (HKLM), H. pylori (HKHP) or S. aureus (HKSA), 500 ng/ml synthetic diacylated lipoprotein (FSL-1), 1 µg/ml ultra-pure lipopolysaccharide (LPS) from E. coli, 1 µg/ml flagellin from S. typhimurium, 20 µg/ml imiquimod (R837), 5 µg/ml ssRNA40, 20 µg/ml ODNs CpG type A, 100 µg/ml poly(I:C), 100 ng/ml TNFα or 1 ng/ml IL-1β. All reagents were purchased from Invivogen except for IL-1β that was purchased from PeproTech. The study trial (clinicaltrials.gov NTC00699868) was approved by the Sud-Méditerranée V Ethics Committee (Comité de Protection des Personnes). Written informed consent was obtained from all patients or their proxies in accordance with the Declaration of Helsinki.
Results

**In vitro induction of B7-H6 transcripts in primary blood cells**

We set up an *in vitro* screening test to investigate whether B7-H6 could be induced on primary blood cells. In this assay, peripheral blood mononuclear cells (PBMCs) were treated with a variety of agents and mRNA was extracted to quantify *B7-H6* transcripts using quantitative RT-PCR. In contrast to reports showing that DNA-damaging agents (such as irradiation, cisplatin, etoposide or fluorouracil) and proteasome inhibitors (such as epoxomicin or MG132) can induce the expression of NKG2D ligands,\(^2\) these treatments had no substantial effect on B7-H6 expression in our protocols (data not shown). However, stimulations of PBMCs with a panel of Toll-like receptors (TLR) agonists induced *B7-H6* transcripts. TLR ligands that are recognized via TLR2, such as Pam3CSK4, HKAL as an example of cell-wall less bacteria, HKLM as an example of Gram\(^+\) bacteria and HKHP as an example of Gram\(^-\) bacteria, zymosan and FSL-1, or via TLR4 such as LPS or via TLR5 such as flagellin, induced a fast expression of *B7-H6* mRNA (Figure 1A). In contrast, the stimulation of PBMCs via endosomal TLRs, such as TLR7 (R837), TLR9 (CpGs) and TLR3 (Poly IC) had very limited impact on *B7-H6* mRNA expression. A noticeable exception was the stimulation of primary cells by ssRNA that triggers a TLR8-dependent pathway and induced *B7-H6* transcripts (Figure 1A). As a control, all TLR stimulation induced *IL-6* transcripts (Supplemental Figure S1). Noteworthy, the pro-inflammatory cytokines TNF\(\alpha\) and IL-1\(\beta\) also induced *B7-H6* transcripts (Figure 1B-C). Overall, the kinetics of *B7-H6* mRNA induction upon TLR and cytokine secretion were quite similar, peaking early between 3 and 12 hours, and returning fast to baseline within 24 hours post-stimulation. Thus, *B7-H6* is one of the early genes that are induced in primary blood human cells upon inflammatory and microbial stimulations *in vitro*. 

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In vitro induction of cell surface B7-H6

To further investigate the regulation of B7-H6 expression, we generated a panel of mouse monoclonal antibodies (mAbs) directed against human B7-H6. Four mAbs were selected on the basis of their selective reactivity with P815.B7-H6, but not with P815.B7-H1 stable transfectants (Figure 2A). As expected, they also reacted with human tumor cell lines constitutively expressing B7-H6 such as HeLa cells (Figure 2A). Two of these mAbs, 4E5.5 and 17B1.3, were blocking the activation of NKp30⁺ reporter cells (DOMsp30) induced by P815.B7-H6 (Figure 2B). Despite their blocking activity, surface plasmon resonance (SPR) analysis showed that they did not inhibit the direct interaction of B7-H6 with NKp30 (Figure 2C). Indeed, the complexes formed between B7-H6 and anti-B7-H6 mAbs not only bound to immobilized soluble recombinant NKp30, but also showed enhanced binding and better stability, as compared to B7-H6 alone (Figure 2C). These data contrast with the inhibition of the interaction between NKp30 and B7-H6 induced by two blocking anti-NKp30 mAbs (Az20 and 1849; Supplemental Figure S2), and prompts further investigation on the mechanisms by which 4E5.5 and 17B1.3 mAbs exert their blocking activity.

The availability of these anti-B7-H6 mAbs allowed us to investigate whether the in vitro inducers of B7-H6 transcripts also led to the cell surface expression of the protein and on which cell type. PBMCs were therefore stimulated with TLR agonists, IL-1β or TNFα for 48 hours, and the cell surface expression of B7-H6 was analyzed by flow cytometry. At steady state, no cell surface expression of B7-H6 on PBMCs could be detected (Figure 3A), consistent with previous findings. At 48 hours, B7-H6 was expressed on the surface of CD45⁺CD14⁺CD19⁻CD3⁻ monocytes upon TNFα, LPS, flagellin and IL-1β stimulation (Figure 3B), consistent with the induction of B7-H6 transcripts in response to these stimulations. The induction of B7-H6 was specific of monocytes, as all others cells from PBMCs remain negative for B7-H6 expression upon in vitro stimulation with TLR ligands or cytokines (data not shown). A low but reproducible induction of B7-H6 protein was seen on monocytes cultured for 48 hours on plastic dishes without any further stimulation (Figure 3B). PolyIC did not lead to the cell surface expression of
B7-H6 on monocytes (Figure 3C), consistent with our transcript expression data. Despite a rapid decline in B7-H6 transcripts (Figure 1A), the cell surface expression of the B7-H6 protein on monocytes was stable up to 48 hours (Figure 3C). The decrease in monocyte viability upon longer period of in vitro culture prevented us to analyze further the kinetics of B7-H6 cell surface expression. CD14+ monocytes were also purified from PBMCs and stimulated in vitro for 48 hours with TLR ligands or pro-inflammatory cytokines. Under these conditions, purified monocytes became B7-H6+, indicating that the induction of B7-H6 on monocytes was the consequence of direct effect of inflammatory or microbial stimulations on these cells (Figure 4A). Even if freshly purified monocytes contain both CD14+CD16− and CD14+CD16+ subsets, upon in vitro culture in the presence or absence of additional stimuli, all monocytes acquired the CD14+CD16+ pro-inflammatory type (Supplemental Figure S3). Although the density of B7-H6 molecules expressed at the surface of monocytes was moderate, it was sufficient to trigger the activation of the NKp30+ DOMsp30 reporter cells. This activation was blocked by the 17B1.3 anti-B7-H6 blocking mAbs (Figure 4B). As a control, no stimulation of the NKp30− parental DO11.10 cells was induced by activated monocytes (Figure 4B). Importantly, IL-1β-stimulated B7-H6+ monocytes triggered autologous NK cells, and this activation was B7-H6-dependent manner, as shown by the inhibitory effect of anti-B7-H6 F(ab’)2 fragments (Figure 4C).

In humans, TLR2 is expressed by monocytes and neutrophils, TLR3 is expressed by BDCA3+ conventional dendritic cells (cDC), TLR5 is expressed by monocytes, neutrophils and cDCs, TLR7 is expressed by monocytes and plasmacytoid DCs (pDCs), TLR8 is expressed by neutrophils and TLR9 by pDCs. The pattern of TLR expression was thus consistent with the selectivity of B7-H6 induction on monocytes, but also prompted us to examine whether neutrophils could express surface B7-H6 upon stimulation. Whereas unstimulated neutrophils do not express cell surface B7-H6, treatment of neutrophils with TNFα, LPS, IL-1β (Figure 4D), HKSA and HKHP (Supplemental Figure S4) leads to a weak but reproducible induction of cell surface B7-H6. As for monocytes, the effect of IL-1β was direct as it occurred on purified neutrophils. Anti-B7-H6 F(ab’)2 fragments were used to ascertain the specificity of anti-B7-H6
mAbs detection on these FcR⁺ myeloid cells (Figure 4D and Supplemental Figure S4), as on monocytes (data not shown).

**In vitro induction of soluble B7-H6**

The availability of a panel of anti-B7-H6 mAbs allowed us to identify that two of them recognize non-overlapping B7-H6 epitopes rendering it possible to set-up a sandwich ELISA against B7-H6 (Supplemental Figure S5). We then investigated whether soluble forms of B7-H6 could be produced *in vitro*. Purified monocytes were activated for 48 hours with LPS or IL-1β and soluble B7-H6 (sB7-H6) was measured in cell supernatants by ELISA. No sB7-H6 was detected in the supernatant of monocytes in absence of stimulation. In contrast, sB7-H6 could be detected in the supernatant of LPS- and IL-1β-stimulated monocytes isolated from a group of healthy individuals (4/9) (Figure 5A). Similarly, neutrophils prepared from another group of healthy individuals and stimulated with LPS or IL-1β also produced sB7-H6 (4/6) (Figure 5B). HKHP was able to induce sB7-H6 from monocytes or neutrophils *in vitro* in some individuals, although stimulated monocytes and neutrophils from all healthy individuals expressed B7-H6 at the surface (Supplemental Figure S4). The mechanisms underlying the heterogeneity between cells from healthy individuals that produce or not detectable amounts of sB7-H6 *in vitro* is still unclear. Irrespective of this issue that needs further investigation, these results reveal that a soluble form of B7-H6 can be produced by neutrophils and monocytes in response to inflammatory and microbial stimuli.

**In vivo expression of B7-H6 during sepsis**

The *in vitro* induction of B7-H6 by TLR ligands and pro-inflammatory cytokines prompted us to analyze the expression pattern of B7-H6 in inflammatory and infectious conditions *in vivo*. To address this point, PBMCs were obtained from a collection of peripheral blood samples prepared from a cohort of 39 patients presenting a systemic inflammatory response syndrome (SIRS) and admitted at the medical intensive care unit (ICU) (Supplemental Table 1). Cells were harvested at
the day of SIRS diagnosis (day 1) and kept frozen. Patients were divided into two categories: 27 patients with sepsis (Sepsis group) and 12 patients with SIRS in absence of detectable microbial infection (SIRS group). Samples from 32 patients out of 39 could be thawed successfully and PBMCs were analyzed for the cell surface expression of B7-H6 on T cells (CD3⁺CD19⁻CD14⁻), B cells (CD3⁻CD19⁺CD14⁻), dendritic cells (CD3⁻CD19⁻CD14⁻DR⁺) and monocytes (CD3⁻CD19⁻CD14⁺). B7-H6 expression was considered positive for samples for which the MFI ratio was strictly above 2. Using these criteria, the cell surface expression of B7-H6 was detected in PBMCs of 15 patients (Figure 6A and Supplemental Table 1). Remarkably, the cell surface expression of B7-H6 was restricted to patients presenting sepsis (Figure 6). Moreover, this expression was selectively observed on CD14⁺CD16⁺ pro-inflammatory type monocytes (Figure 6A), consistent with our *in vitro* data (Figure 4A and Supplemental Figure S3). As expected, no cell surface expression of B7-H6 was detected on monocytes from healthy control individuals (Figure 6C). The absence of neutrophils in frozen PBMC preparations prevented us to analyze whether surface B7-H6 could be detected on these cells. sB7-H6 was also detected in the serum of 10/39 patients and restricted to sepsis group (Figure 6D). No sB7-H6 was detected in sera obtained from other SIRS patients or healthy individuals (Figure 6D). Overall, 65% of patients with sepsis had circulating B7-H6⁺CD14⁺CD16⁺ pro-inflammatory type monocytes, when no B7-H6⁺ PBMCs could be detected in other SIRS patients or healthy individuals. No difference was observed between sepsis patients presenting Gram⁻ infections (*A. xylosoxidans*, *E. coli*, *E. aerogenes*, *K. pneumonia* and *P. aeruginosa*) or Gram⁺ (*S. aureus*, *S. pneumonia* and *S. mitis*) infections. In contrast, serum sB7-H6 was detected in 37% of sepsis patients, and restricted to patients with Gram⁻ infection (Figure 6E and Supplemental Table 2). Consistent with these results, Gram⁻ bacteria (e.g. HKHP) are much more prone than Gram⁺ bacteria (e.g. HKSA) to induce sB7-H6 *in vitro* (Supplemental Figure S4). Most Gram⁻ sepsis patients who had detectable levels of serum sB7-H6 at day 1 also had circulating B7-H6⁺CD14⁺CD16⁺ pro-inflammatory monocytes on the same day (Supplemental Table 1).
Despite the small size of our cohort of patients, we analyzed the potential association of B7-H6 expression and the clinical outcome of ICU patients. The overall day 30 mortality in ICU patients was 23.1%. We observed a higher mortality in sepsis patients with membrane B7-H6 (mB7-H6+) expression as compared to patients lacking mB7-H6+ cells (mB7-H6−) (Figure 6F). In particular, no death was observed when mB7-H6 expression was negative in patients with Gram− sepsis, as compared to 44% death in patients expressing mB7-H6.

Analysis of sB7-H6

We took advantage of the substantial quantities of sB7-H6 present in patient sera to document the nature of sB7-H6. In a first series of experiments, patient sera were subjected to three rounds of centrifugations. The pellets obtained at each round were analyzed by ELISA for the presence of sB7-H6. sB7-H6 was associated with the material that sedimented at 110,000 g, suggesting that sB7-H6 was included in membrane vesicles that were present in the serum, such as exosomes (Figure 7A). To further dissect this point, sB7-H6+ patient sera were analyzed for the presence of B7-H6+ membrane vesicles using sequential low-speed centrifugations and high-speed centrifugations followed by a 0.22 μM filtration as described previously. After incubation of the filtered pellets with latex beads, we detected that sB7-H6+ patient sera contained filtered pellet material that was reactive with CD63, CD81 and B7-H6 mAbs (Figure 7B). CD63 and CD81 are classically considered as markers of exosomes, suggesting that B7-H6 can be associated with these membrane vesicles in the serum of sepsis patients. Incubation of primary NK cells with the sB7-H6+ pellets that sedimented with the exosomal fraction impaired the staining of NK cells with anti-NKp30 mAbs, whereas sB7-H6− pellets had no effect (Figure 7C). In addition, sB7-H6+ pellets also inhibited the NKp30-dependent activation of reporter cells induced by B7-H6+ K562 cells as efficiently as the blocking 17B1.3 anti-B7-H6 mAbs (Figure 7D). Interestingly, recombinant sB7-H6 had no effect on NKp30 cell surface expression and on NKp30-dependent cell activation over a large range of concentrations (data not shown). The available anti-NKp30 mAbs used to stain NK cells were blocking anti-NKp30 reagent, making it impossible to
formally show that sB7-H6⁺ pellets down-regulated NKp30 cell surface expression or merely masked surface NKp30. Nevertheless and irrespective of the formal demonstration that sB7-H6 was included in serum exosomes, the impact of sB7-H6⁺ serum pellets on NKp30 staining and cell activation contrasted with the failure of recombinant sB7-H6 to do so. Of note, our data do not exclude that a form of sB7-H6 was not associated with exosomes. However, there are no described alternative spliced products of B7-H6, which could correspond to a soluble form lacking the transmembrane domain. Moreover, we could not detect using our panel of mAbs a shed form of B7-H6 which could be released from the plasma membrane by proteolytic cleavage (data not shown).
Discussion

B7-H6 is the most recently described member of the B7 family of cell surface immunoreceptors.12,13,17-21 Thus far, the expression of B7-H6 has been shown to be restricted to tumor cells and absent from normal hematopoietic cells from healthy individuals at steady-state.12,17 It is well known that B7 family members are induced on myeloid cells upon stimulation with infectious and pro-inflammatory stimuli.22-25 However, no data have been reported on the mechanisms leading to the induction of B7-H6. We have shown here that B7-H6 transcripts, B7-H6 cell surface expression and sB7-H6 can be induced in inflammatory conditions in vitro and in vivo. Of note, B7-H6 could not be detected at the surface of monocyte-derived DC (moDC).12 Functional studies demonstrated that NKp30 is crucial for the interaction of NK cells with moDC,26 while it seems dispensable in the crosstalk with macrophages.27 Thus, two cell types both derived from monocytes would either express a ligand different from B7-H6 (moDC) or, perhaps, lack NKp30 ligand expression (macrophages). Irrespective of this issue, our data indicate that B7-H6 expression is not limited to tumor cells, and reveal that non-transformed cells could be recognized as targets of NK cells through B7-H6/NKp30 interactions. They further support the concept introduced earlier for the induction of NKG2D ligands as which the ligands for NK cell activating receptors are silenced on normal cells and induced in various conditions of cellular stress, such as infection, inflammation and cancer. There are similarities and differences between the conditions that lead to NKG2D ligands and B7-H6 expression. On the differences, we could not detect B7-H6 induction on the surface of cells treated with DNA damaging agents or proteasomes inhibitors that have been shown to induced cell surface NKG2D ligands.28,29 On the similarities, TLR signaling has been shown to up-regulate transcription and expression of NKG2D ligands, such as retinoic acid early inducible-1 (RAE-1) family members in mouse macrophages30 and MICA on human macrophages.31,32 These data have led to propose some molecular basis for the cross-talks between NK cells and monocytes/macrophages during innate immune response to infections. Interactions between NK and monocytes/macrophages have been
indeed documented in several inflammatory and infections conditions. As NKp30 is expressed by NK cells and B7-H6 only binds to NKp30, our present data on the induction of B7-H6 on inflammatory monocytes and neutrophils support this idea. The dissection of the mechanisms involved in these conditions is particularly relevant to sepsis, the control of which represents an important unmet medical need.

Sepsis is a clinical syndrome that complicates severe infection, and is one of the leading causes of admission to ICU with mortality rates ranging from 30% to more than 50%. The host-response of these patients, include a first phase of SIRS, characterized by an exacerbated inflammatory response, rapidly followed by a profound alteration of immunity, referred as to compensatory anti-inflammatory response syndrome (CARS). This acquired immunoparalysis is thought to render the patients more susceptible to nosocomial infections, and to lead to increased morbidity and mortality. NK cells are a major source of interferon (IFN)-γ, a potent inflammatory cytokine, and early depletion of NK cells improved survival in sepsis models in the mouse. We also recently observed that both NK cell cytotoxicity and IFN-γ production were decreased in sepsis, consistent with similar results obtained in mice. Considering the sequence of opposite events that are at work during sepsis, NK cells might have a dual role in sepsis, first contributing to the amplification of the inflammatory response during the early steps of SIRS and then, impaired during CARS and thus participating to its detrimental consequences.

Our data on B7-H6 expression during inflammation provide a novel perspective on the link between microbial infection and NK cell activation. First, B7-H6 was selectively induced on the surface of CD14+CD16+ pro-inflammatory monocytes and neutrophils, two types of cells which play a central role in the onset and amplification of inflammation. In particular, CD14+CD16+ monocytes are described as the main producers of inflammatory cytokines such as TNFα and IL-1β in response to LPS. Several studies have reported that CD14+CD16+ monocytes are found in larger numbers in the blood of patients with acute inflammation and infectious diseases such as sepsis, tuberculosis and rheumatoid arthritis. Second, B7-H6 could be produced as a soluble form in vitro by activated monocytes and neutrophils, and in vivo in a
group of sepsis patients. Soluble forms of B7 family members as CD80, CD86, B7-H1, B7-H2, B7-H3 and B7-H4 have been detected in the serum of patients with cancer and inflammatory conditions.\textsuperscript{46-51} Although in several instances soluble B7 receptors have been shown to serve as decoy molecules to block the function of their receptors, the biological roles of these molecules remain to be dissected in detail. The present description of cell surface and soluble B7-H6 confirms the general propensity of the members of the family to be induced in inflammatory conditions. sB7-H6 present in the serum of sepsis patients impaired the binding of anti-NKp30 mAbs to NKp30 and NKp30-dependent cell activation. Importantly, recombinant sB7-H6 was unable to impact on NKp30 expression and signaling. Thus, sB7-H6 present in patient serum is different from recombinant sB7-H6. These data support the fact that serum sB7-H6 was found in exosomal fractions and thus could be exposed as multimers with higher avidity that monomeric recombinant sB7-H6. It is however remarkable and still puzzling that serum sB7-H6 was restricted to patients with Gram infection, while no difference was observed between sepsis patients presenting Gram or Gram+ infections for mB7-H6 expression. A possible explanation could be that blood monocytes recognize Gram+ bacteria through TLR2 and TLR4 while Gram+ bacteria are recognized by TLR2. In response to ligand binding to TLR4, two major signaling pathways are activated. One pathway depends on myeloid differentiation marker 88 (MyD88) and is common to TLR2 and TLR4, whereas the other depends on Toll-IL-1 receptor (TIR) domain-containing adaptor inducing interferon-β (TRIF) restricted to TLR4.\textsuperscript{52} Consequently the induction of mB7-H6 could be dependent of MyD88, while the induction of sB7-H6 could be dependent of TRIF. This issue needs further investigation.

During an immune response to infection, the outcome of NK cell-myeloid cell interactions through NKp30/B7-H6 interaction could be two-fold according to the membrane bound or soluble form of B7-H6. In one hand, 65% of patients with sepsis had circulating pro-inflammatory type monocytes expressing mB7-H6, when these cells could not be detected in other SIRS patients or healthy individuals. This suggests that NK cell activation induced by B7-H6+ monocytes could occur in these patients via NKp30 interaction. The highest mortality of
patients with membrane bound B7-H6 would suggest a negative role for NK cells in sepsis, consistent with data in the mouse.\textsuperscript{36,37} In the other hand, we also show that sB7-H6 which block mB7-H6/NKp30 functional interaction, was detected in the serum of sepsis patients. We observed a trend associating sB7-H6 with higher patient mortality (data not shown). Thus, NK cell inhibition via sB7-H6 production would also be detrimental for the patient outcome. Based on these data, we thus propose a model as which the activation of NK cells via mB7-H6 contributes to inflammation during SIRS, but then sB7-H6 might play a negative role during CARS. This possibility would explain how two opposing phenomena, (i.e the expression of mB7-H6 that activates NK cells and the production of sB7-H6 that impairs NK cell activation), could have both deleterious impact on patient survival during sepsis. Our data thus prompt to perform the immunomonitoring of cell surface B7-H6 and sB7-H6 in a large of cohort of sepsis patients over the kinetics of their clinical outcome to test whether mB7-H6 and/or sB7-H6 can help in stratifying individuals at risk of developing bad outcomes.
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Authorship contribution

J. M. and E. V. designed and analyzed the experiments and wrote the paper; J. M. performed most of the experiments; M. B. generated the anti-B7-H6 mAbs; C. C., C. P., C. F, F. V and J. M. performed and analyzed experiments on human patients and control individuals. L. C, J-M. F., G. T and L. P. were responsible for the clinical studies and the statistical analysis. D. C. and S. U. provided expression data and critical comments on the report.

Conflict of interest statement

E.V. is a cofounder and shareholder of Innate-Pharma.
References


Figure Legends

**Figure 1. In vitro induction of B7-H6 transcripts.** B7-H6 mRNA is up-regulated in PBMCs during activation by (A) TLR ligands, (B) TNFα or (C) IL-1β. Control cells were treated with medium only. Data show fold induction of B7-H6 mRNA in treated cells as compared to untreated control cells at the indicated time points. Data correspond to a pool of three independent experiments.

**Figure 2. Characterization of anti-B7-H6 mAbs.** (A) P815.B7-H1, P815.B7-H6 or HeLa cells were stained with 4E5.5, 9G9.2, 10E2.9 or 17B1.3 anti-B7-H6 mAbs and analyzed by flow cytometry. Mouse IgG1 (mIgG1) was used as isotype control. Graphs are representative of at least three experiments. (B) NKp30⁺ DOMsp30 reporter cells were co-cultured with P815.B7-H6 cells in presence or absence of anti-B7-H6 mAbs. DOMsp30 cell activation was determined by evaluating IL-2 production in the co-culture supernatant in a standard CTLL-2 survival assay. Data are representative of three independent experiments. a.u: arbitrary units. ***P<0.001. (C) SPR analysis: superimposed sensorgrams showing the injections onto NKp30 chip of soluble recombinant B7-H6 alone or pre-incubated with anti-B7-H6 mAbs. Sensorgrams were normalized in the Y axis and aligned in the X axis at the end of injection. Sensorgrams are representative of two independent experiments.

**Figure 3. In vitro induction of B7-H6 cell surface expression on monocytes.** Flow cytometric analysis of CD45⁺CD14⁺CD19⁻CD3⁻ monocytes, gated from freshly isolated PBMCs, (A) left untreated or (B) after stimulation for 48 hours with TNFα, LPS, flagellin or IL-1β. B7-H6 expression was analyzed by flow cytometry with directly conjugated 17B1.3 anti-B7-H6 mAbs (black histograms) or mIgG1 isotype control (gray histograms). Data are representative of at least three independent experiments. MFI stim represents the value of the Mean Fluorescence Intensity (MFI) obtained with anti-B7-H6 mAbs minus the MFI obtained with mIgG1 isotype control. (C)
PBMCs were left untreated or treated with PolyIC, TNFα, LPS, flagellin or IL-1β at indicated time points. B7-H6 cell surface expression was assessed by flow cytometry and fold change in B7-H6 expression was quantified by dividing the MFI of treated samples by that of untreated cells at each time points. Data correspond to a pool of at least three independent experiments. Statistical analyses were performed using two-way ANOVA test with Bonferroni correction. *P<0.05; **P<0.01; and ***P<0.001.

Figure 4. In vitro induction of B7-H6 cell surface expression on purified myeloid cells and NKp30-dependent cell activation induced by B7-H6+ monocytes. (A) Flow cytometric analysis of B7-H6 cell surface expression on freshly isolated monocytes stimulated for 48 hours with TNFα, LPS or IL-1β. B7-H6 expression was analyzed by flow cytometry with directly conjugated 17B1.3 anti-B7-H6 mAbs (black histograms) or mIgG1 isotype control (gray histograms). Data are representative of at least three independent experiments. MFI stim represents the value of the Mean Fluorescence Intensity (MFI) obtained with anti-B7-H6 mAbs minus the MFI obtained with mIgG1 isotype control. (B) NKp30+ DOMsp30 reporter cells (NKp30+) or NKp30− DO11.10 control cells (parental cells) were co-cultured with unstimulated or IL-1β-stimulated monocytes in the presence of anti-B7-H6 mAbs (17B1.3) or mIgG1 isotype control. DOMsp30 cell activation was determined by evaluating IL-2 production in the co-culture supernatant in a standard CTLL-2 survival assay. Data are representative of three independent experiments. a.u: arbitrary units. ***P<0.001. (C) Autologous NK cells were co-cultured with unstimulated or IL-1β-stimulated monocytes (E:T; 1:4) in the presence of anti-B7-H6 (17B1.3) F(ab’)2 fragments. NK cell activation was determined by evaluating the percentage of CD107 positive NK cells. Data correspond to a pool of six independent experiments. *P<0.05; **P<0.01; and ****P<0.0001. (D) Flow cytometric analysis of B7-H6 expression on freshly isolated CD24+CD14−HLA DR− neutrophils left untreated or stimulated with TNFα, LPS or IL-1β for 24 hours. B7-H6 expression was analyzed by flow cytometry with F(ab’)2 fragments of the B7-H6 specific 17B1.3 mAbs followed by APC conjugated anti-mouse IgG (black histograms) or
with APC conjugated anti-mouse IgG alone (control gray histograms). Data are representative of at least three independent experiments. MFI stim represent the value of the MFI obtained with anti-B7-H6 F(ab)’2 minus the MFI obtained with the control.

**Figure 5. In vitro induction of sB7-H6 from human monocytes and neutrophils.** (A) Freshly isolated monocytes or (B) freshly isolated neutrophils were treated with LPS or IL-1β for the indicated time points. Concentrations of sB7-H6 were measured in the cell supernatant by ELISA. Each symbol represents one individual donor. Graph represents 9 healthy individuals donors for monocytes and 6 healthy individuals donors for granulocytes.

**Figure 6. In vivo expression of B7-H6 on inflammatory monocytes during sepsis and association of B7-H6 and sepsis conditions.** Flow cytometric analysis of B7-H6 expression on CD45+CD14+CD19−CD3− monocytes, gated from PBMCs of (A) sepsis patients, (B) SIRS patients and (C) healthy donors. Cells are stained with CD14, CD16 mAbs and directly conjugated 17B1.3 anti-B7-H6 mAbs (black histograms) or mIgG1 isotype control (gray histograms). Data are representative of 15 sepsis patients (23 patients in total), 9 SIRS patients and 14 healthy donors. (D) Concentrations of sB7-H6 in the sera of sepsis patients, SIRS patients or healthy donors were measured by ELISA. Statistical analyses were performed using Student t test. (E) Sepsis patients were stratified according to the nature of their sepsis (induced by Gram⁺LPS⁻ or Gram⁻LPS⁺ bacteria), and concentrations of sB7-H6 were measured by ELISA. Statistical analyses were performed using Student t test. (F) Kaplan Meier curves of survival probability obtained by segregating the whole cohort of ICU patients into two groups according to the expression of B7-H6 cell surface expression. The number of specimen is indicated on the graph. mB7-H6⁺: B7-H6 positive cells; mB7-H6⁻: B7-H6 negative cells. Statistical analyses were performed using Pearson’s X2 test.
Figure 7. Characterization of sB7-H6 isolated from patient sera. (A) Concentrations of sB7-H6 were measured in the pellet at each round of three sequential centrifugations at 1200 g, 12,000 g and 110,000 g of B7-H6\(^+\) patient sera. Data correspond to a pool of three independent experiments. (B) Flow cytometric analysis of pellets purified from serum of two sB7-H6\(^+\) patients and one control individual. After purification, pellets were complexed to latex beads and stained with the indicated mAbs (black histograms), histograms obtained with isotype controls are in gray. (C) Flow cytometric analysis of NKp30 cell surface expression on freshly isolated NK cells stained with mIgG1 isotype control (gray histograms) or NKp30 mAbs in presence of 5 \(\mu\)g of sB7-H6\(^+\) pellet (dashed line) or 5 \(\mu\)g of sB7-H6\(^-\) pellet (black line). Data are representative of three independent experiments. (D) NKp30\(^+\) DOMsp30 reporter cells or NKp30- DO11.10 control cells (parental cells) were co-cultured with K562 cells that constitutively express B7-H6, in the presence of anti-B7-H6 mAbs (17B1.3), mIgG1 isotype control, 5 \(\mu\)g of pellet from serum of two sB7-H6\(^+\) patients or 5 \(\mu\)g of pellet from serum of two sB7-H6\(^-\) patients. DOMsp30 cell activation was determined by evaluating IL-2 production in the co-culture supernatant in a standard CTLL-2 survival assay. Data are representative of three independent experiments. a.u: arbitrary units.
Figure 2

A

B7-H6

P815.B7-H1  P815.B7-H6  HeLa

17B1.3
10E2.9
9G9.2
4E5.5
mlgG1

B

IL-2 production by reporter cells (CT2L2 viability, a.u.)

mAbs in the coculture

None  mlgG1  4E5.5  9G9.2  10E2.9  17B1.3

C

RU (Resonance Units)

0  5  10  15  20  25  30  35  40  45

0  50  100  150  200  250

Time (s)

B7H6+isotype control
B7H6+17B1.3
B7H6+4E5.5
B7H6

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Figure 3

A
Untreated

B
Without stimulation  TNFα  LPS  Flagellin  IL-1β

C
PolylC  TNFα  LPS  Flagellin  IL-1β

Time (h)  Time (h)  Time (h)  Time (h)  Time (h)
Figure 5

A Monocytes

Without stimulation

LPS

IL-1β

B Neutrophils

Without stimulation

LPS

IL-1β
Figure 6

A Sepsis patients (15/23)

B SIRS patients (9/9)

C Healthy donors (14/14)

D

E

F

Log Rank Chi² = 3.88; p = 0.049

Healthy SIRS Sepsis

Log Rank Chi² = 3.88; p = 0.049

Gram+ Gram-

n=12 n=27

n=9 n=18

- mB7H6- 

- mB7H6+

n=17 n=15
Induction of B7-H6, a ligand for the Natural Killer cell activating receptor NKp30, in inflammatory conditions

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