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Letter to the Editor

CTLA-4 regulates human Natural Killer cell effector functions

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To The Editor

Cytotoxic T lymphocyte antigen 4 (CTLA-4) is a member of a family of immunoglobulin-related receptors that includes CD28, Inducible T-cell costimulator (ICOS), Programmed cell death protein 1(PD-1), B- and T-lymphocyte attenuator (BTLA) and T cell immunoreceptor with Ig and ITIM domains (TIGIT) [1, 2]. These receptors have been mainly studied in T cells and have been characterized by stimulatory (CD28, ICOS) or inhibitory functions (CTLA-4, PD-1, BTLA and TIGIT) in T cell biology [1, 2]. CTLA-4 and CD28 both interact with the B7 molecules CD80 and CD86, although with opposing effects (CTLA-4: inhibitory; CD28: activating) [1, 2]. Recently, monoallelic mutations in CTLA-4 leading to CTLA-4 haploinsufficiency were identified in a complex syndrome of immunodeficiency and immune dysregulation [3, 4]. While the CD28/B7 axis has been studied in human and murine Natural Killer (NK) cells [5, 6], data on the expression and function of CTLA-4 in human NK cells are largely lacking. We report for the first time that CTLA-4 is expressed in activated human NK cells and that CTLA-4 haploinsufficient human NK cells show defective effector functions, although in the absence of maturational alterations.

To date, CTLA-4 expression has been shown to be up-regulated in activated murine NK cells [7]. Thus, we decided to investigate whether human NK cells expressed CTLA-4 upon activation as well. As shown in Fig. 1A, B and C, CTLA-4 expression in human NK cells from healthy controls (HDs) can be induced upon activation with the maximum levels observed upon combined Interleukin-2 (IL-2), Interleukin 12 (IL-12) and Interleukin 18 (IL-18) stimulation, a finding that has not been reported before. On the contrary, CTLA-4 haploinsufficient NK cells failed to up-regulate CTLA-4 upon stimulation, as shown in Fig. 1B and C. Data shown depict NK cells from two affected patients (Pt1 and Pt5) due to limited availability of biological material from the other patients. In order to evaluate the possible role of CTLA-4 on human NK cell maturation and effector functions, NK cells from patients with CTLA-4 haploinsufficiency were then evaluated. Five CTLA-4 haploinsufficient patients harboring different CTLA-4 mutations were included in this study (Supplemental Table 1). Peripheral NK cell maturation did not show significant perturbations when compared to age-matched healthy controls (Supplemental Results and Supplemental Fig. 1A). We then went on to investigate whether the lack of CTLA-4 in human NK cells could affect their effector functions, namely cytotoxicity and IFN-γ production. Baseline degranulation against the human erythroleukemia cell line K562, measured as CD107a expression level of resting NK cells, was similar between healthy controls (HDs) ($n = 10$) and patients (Pts) ($n = 5$) (Fig. 1D). However, upon IL-2 stimulation, patients' NK cell degranulation against the human erythroleukemia cell line K562 was significantly reduced as compared to healthy controls (Fig. 1D). This novel finding underlines a degranulation defect of CTLA-4 haploinsufficient NK cells that may explain the increased susceptibility to viral infections of affected patients [3, 4, 14]. IL-2 dependent NK cell proliferation, as defined by Ki67 staining, in a CTLA-4 haploinsufficient patient (Pt), was similar to that of an age matched healthy control (HD) (Supplemental Fig. 1B), suggesting that the IL-2 pathway in NK cells is functionally intact in CTLA-4 haploinsufficiency. We then investigated whether IFN-γ production, a biological hallmark of NK cells, was affected in CTLA-4 haploinsufficiency. IFN-γ production was thus evaluated by patients' and healthy controls' NK cells before and after simultaneous stimulation with IL-12 and IL-18. As expected, baseline (without stimulation) IFN-γ production was virtually absent both in healthy controls (HDs) ($n = 10$) and affected patients (Pts) ($n = 5$) (Fig. 1E). Interestingly, upon combined IL12 and IL-18 stimulation, IFN-γ production from patients' NK cells was significantly reduced when compared to healthy controls (Fig. 1E), a finding that has not been reported before. These novel results underline a critical role for CTLA-4 in human NK cell IFN-γ production upon *in vitro* combined IL-12 and IL-18 stimulation.

CTLA-4 represents an important immunological check-point [1, 2, 12, 13]. Monoallelic mutations in CTLA-4 cause a complex disorder with immunodeficiency, infections (bacterial and viral), autoimmunity and immune disregulation [2, 3, 14]. In addition, CTLA-4 blocking antibodies have been successfully used in murine cancer models and are now also available in certain forms of human tumors [1, 12, 13]. Of interest, both mice and patients treated with blocking CTLA-4 antibodies develop autoimmune manifestations, resembling those of CTLA-4 haploinsufficient patients [12, 13]. NK cells have been implicated in autoimmune diseases both in animal models and in humans, suggesting an important interplay between innate and adaptive immunity for the development and/or maintenance of autoimmunity [8, 9, 10]. The novel NK cell defects observed in CTLA-4 haploinsufficiency may thus contribute both to the increased susceptibility to viral infections, in

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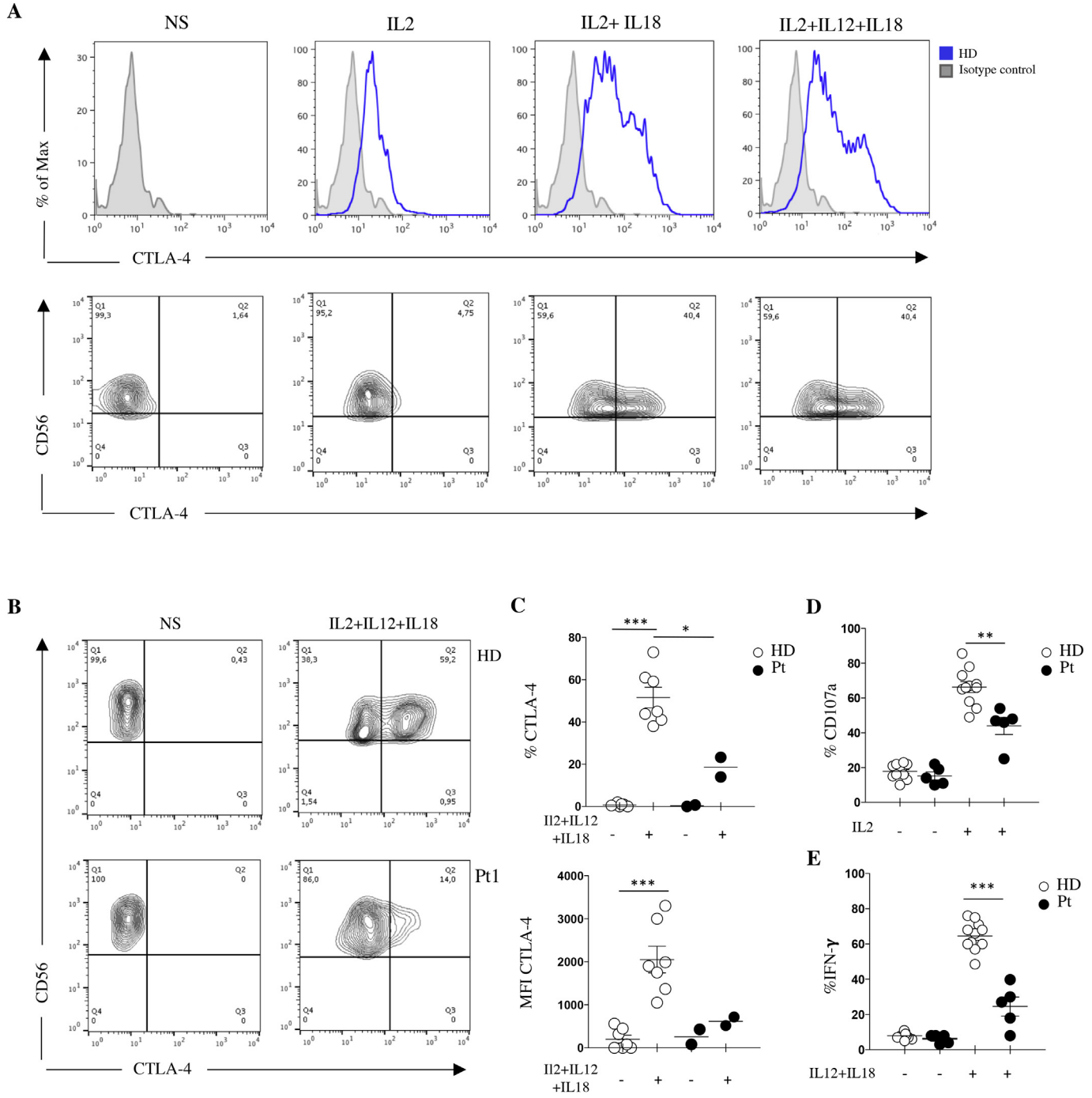

Fig. 1. CTLA-4 and human NK cells. A. CTLA-4 expression in human $CD45^+CD3^-CD14^-CD20^-CD56^+$ NK cells from a healthy control (HD) upon stimulation with IL-2, IL-12 + IL18 and IL-2 + IL12 + IL18 (upper panel: histograms; lower panel: dot plots). B. CTLA-4 expression in human $CD56^+$ NK cells from a CTLA-4 haploinsufficient patient (Pt1) and an age-matched healthy control (HD) at the resting state (NS) and upon stimulation with IL-2 + IL-12 + IL18. **C.** Summary of CTLA-4 expression in activated human $CD45^+CD3^-CD14^-CD20^-CD56^+$ NK cells from 7 healthy controls (HDs) and 2 CTLA4-mutated patients (Pts); data are expressed in terms of percentages (upper panel) and mean fluorescence intensity (MFI) (lower panel). Data were obtained from single experiments performed in triplicate and statistical analysis was performed using the *t*-student test (* = $p < 0,05$; ** = $p < 0,005$; *** = $p < 0,0005$; Scatter plots show mean +/-SD. D. Summarized data for CD107a up-regulation in NK cells upon IL-2 stimulation against the human erythroleukemia cell line K562 from healthy controls (HDs) ($n = 11$) and CTLA-4 haploinsufficient patients ($n = 5$). E. Summarized data for IFN-γ production (shown as percentages of positive cells) from healthy controls (HDs) (n = 11) and CTLA-4 haploinsufficient patients (n = 5). before and after combined stimulation with IL-12 and IL-18. Statistical analysis was performed using the t-student test (* = $p < 0,05$; ** = $p < 0,005$; *** = $p < 0,0005$). Scatter plots show mean +/-SD.

particular EBV and CMV [14] and to autoimmune phenomena observed in affected patients [3, 4, 14]. Of note, blocking of PD-1, another important immune check-point [11], was recently shown to impair NK cell effector functions, further suggesting a novel biological role for these immune check-points, namely CTLA-4 and PD-1, in NK cell biology.

Taken together, our data show for the first time that CTLA-0.4 is expressed in human NK cells and that CTLA-4 haploinsufficiency in humans is associated with defective NK cell effector functions, *i.e.* cytotoxic machinery and IFN-γ production, in the presence of normal peripheral NK cell maturation, with important implications in clinical

conditions where CTLA-4 is lacking and potentially in clinical settings where CTLA-4 is used as a therapeutic target.

Supplementary data to this article can be found online at https://doi.org/10.1016/j.clim.2018.06.010.

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Disclosure of conflicts of interest

The authors declare no conflict of interest.

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