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Impaired antigen presentation and potent phagocytic activity identifying tumor-tolerant human monocytes

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ABSTRACT

Monocyte exposure to tumor cells induces a transient state in which these cells are refractory to further exposure to cancer. This phenomenon, termed "tumor tolerance", is characterized by a decreased production of proinflammatory cytokines in response to tumors. In the past, we found that this effect comprises IRAK-M up regulation and TLR4 and CD44 activation. Herein we have established a human model of tumor tolerance and have observed a marked down-regulation of MHCII molecules as well as the MHCII master regulator, CIITA, in monocytes/macrophages. These cells combine an impaired capability for antigen presentation with potent phagocytic activity and exhibit an M2-like phenotype. In addition circulating monocytes isolated from Chronic Lymphocytic Leukemia patients exhibited the same profile as tumor tolerant cells after tumor *ex vivo* exposition.

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1. Introduction

Monocytes/macrophages (M∅s) are involved in the immune response to tumors as they present tumor associated antigens and act as cytotoxic effector cells [1]. There is considerable evidence that cancer cells induce the expression of proinflammatory cytokines and reactive oxygen species in M∅s [2]. This kind of cells is the major inflammatory component of tumor infiltrates and is a prominent part of the inflammatory circuits that promote tumor progression [3]. Clinical data suggest that the protective influence of Tumor-Associated Macrophages (TAMs) is depressed in cancer patients [4,5]. Tumors counteract the cytotoxic and proinflammatory activities of TAMs [6,7]. This in turn leads to the down regulation of TNFα, IFNγ, IL12p40 and IRAK-1 expression [8]. In addition, there is a faster up regulation of the negative inflammation controller, IRAK-M [2]. Published data also indicate that IRAK-M$^{-/-}$ dendritic cells increase the proliferation and activation of Ag-specific T cells [9]. It has been demonstrated that the expression of this pseudokinase controls the "tumor tolerance" of TAMs and, subsequently, the innate immune system [2,10]. In summary, M∅s have a dual function in their interaction with neoplastic cells, since they can express activities that prevent the establishment and spread of tumor cells and, simultaneously, have functions to support tumor growth and dissemination [6,11–13]. This ambivalent relationship reflects the elevated functional plasticity of macrophages, which are able to express different functional programs in response to different micro environmental signals, such as those exemplified in the M1-M2 paradigm of macrophage polarization [13,14–16]. While M1 macrophages are potent effector cells that kill microorganisms and tumor cells, they also produce copious amounts of proinflammatory cytokines; M2 macrophages tune inflammatory responses and adaptive Th1 immunity, scavenge debris as well as promote angiogenesis, tissue remodeling, and repair [17]. In established progressing tumors, TAMs generally fail to express antitumor activity and have properties of alternatively activated or M2-like cells, oriented to the promotion of tissue remodeling, angiogenesis, and taming of protective adaptive immunity [16]. Moreover, tumor cells produce molecules that induce a M2 phenotype in TAMs [2]. It has been

Abbreviations: M∅s, monocytes/macrophages; CLL, Chronic Lymphocytic Leukemia; TAMs, Tumor Associated Macrophages.

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shown that M1 macrophages present an IL-12^{high}/IL-23^{high}/IL-10^{low} phenotype, while M2 macrophages exhibit the complementary IL-12^{low}/IL-23^{low}/IL-10^{high} phenotype with a variable signal-dependent capacity to produce inflammatory cytokines [18]. Tumor-tolerant M∅s seem to exhibit a M2 phenotype [19].

In this paper we established a model of human tumor-tolerant M∅s and studied their main characteristics. We confirmed that they share an IL-12^{low}/IL-23^{low}/IL-10^{high} phenotype with M2 cells, as well as an up regulation of IRAK-M. We also observed an evident down-regulation of HLA class II molecules and MHCII master regulator CIITA that suggest a poor capability for antigen presentation. Lymphocyte proliferation assays corroborated this feature of tumor-tolerant M∅. In addition, these cells also exhibited a high phagocytic ability. These data were sustained by results with monocytes isolated from fifteen non-treated Chronic Lymphocytic Leukemia patients. Our results provide evidences of a specific phenotype in tumor-tolerant cells, characterized by an increased potential for phagocytosis, along with an impaired presentation of antigens via MHC molecules that could contribute to immunity impairment.

2. Material and methods

2.1. Reagents

Anti-CD3-PE, anti-HLAII-DQ-FITC and anti-HLAII-DR-APC were purchased from Becton Dickinson (San Jose, CA, USA); anti-CD14-FITC was from Miltenyi Biotec (Auburn, CA, USA) anti-CD1a-FITC, anti-CD16b-FITC, and anti-CD89-FITC (Serotec). Anti-β-actin and anti-TNFα was from Santa Cruz Biotechnologies (Santa Cruz, CA, USA). Anti-IRAK-M was acquired from Chemicon International (Temecula, CA, USA).

2.2. Patient and healthy controls

We studied fifteen patients from Hospital Universitario Infanta Sofía diagnosed with Chronic Lymphocytic Leukemia (CLL, $n = 15$), according to the Classification of Tumors of the Haematopoietic and Lymphoid Tissues by the World Health Organization [20]. Also eight healthy volunteers, age matched, were included. Blood samples were collected in EDTA and delivered at room temperature. Written informed consent was obtained from all subjects enrolled. The local Ethics Committee approved this study.

2.3. PBMC isolation and cell culture

PBMC were isolated from buffy coats by centrifugation on Ficoll-Hypaque Plus (Amersham Biosciences; Piscataway, NJ, USA) according to the manufacturer's indications. Cells (monocytes and lymphocytes) were cultured following our previous protocol [2,21–24]. Primary cultures of human fibroblasts were obtained as previously reported [2].

2.4. Tumor cell lines

The human cell lines A431 (human epidermal carcinoma), HeLa (human cervical carcinoma) Bxpc3 (human pancreatic adenocarcinoma) and SKBR3 (human breast adenocarcinoma) were provided by the American Type Culture. All cell lines were tested for mycoplasma and LPS contamination.

2.5. Co-cultures and cell isolation

Human monocytes were co-cultured with different tumor cell lines at a ratio of 1:0.3 (M∅s:tumor cells) for the times indicated in Results section. After incubation, pooled cells were harvested and stained with an anti-CD14 Ab conjugated to magnetic beads from Miltenyi Biotec (Auburn, CA, USA). CD14-positive cells (CD14+) were isolated using Automacs System and recovered for further assays (see the scheme in Supplementary Fig. 1A).

2.6. ELISA and Western blot analysis

IL6 and IL10 levels in supernatants were determined with ELISAs (Bender MedSystems). The Western blot analysis was performed following previously reported protocol [2,21–24].

2.7. RNA isolation and cDNA synthesis

Cells were washed once with PBS and their RNA was isolated and cDNA synthesized as previously reported [2,21–24].

2.8. mRNA quantitation

Gene expression levels were analyzed by real-time quantitative PCR (Q-PCR) using the LightCycler system from Roche Diagnostics, a QuantiMix Easy SYG kit from Biotools (Madrid, Spain) and specific primers. Results were normalized to the expression of the β-actin gene, and the cDNA copy number of each gene of interest was determined using a seven-point standard curve as we described before [2,21–24].

2.9. Flow cytometric analysis

Surface proteins were detected using the specific antibodies indicated above, as previously described [24].

2.10. Phagocytosis and bacteria killing assays

We followed protocols previously described [25,26].

2.11. Proliferation assay

We followed a protocol previously described [27,28].

2.12. Data analysis

The number of experiments analyzed is indicated in each figure. Data were collected from a minimum of three experiments to calculate the mean ± SD. The statistical significance was calculated using the unpaired Student's test and differences were considered significant at p values < 0.05 using Prism 5.0 software (GraphPad, San Diego, CA, USA). In other experiments an ANOVA analysis was followed by a Turkey test.

3. Results

3.1. Tumor cells induce tolerance of human monocytes/macrophages in vitro

During a tumor process, monocytes/macrophages (M∅s) are one of the primary effectors of innate immunity. However, after a first encounter with cancer cells, M∅s enter a refractory state termed "tumor tolerance", in which further responses to a tumor cell are severely blunted [2,10,29]. Herein we study the main features of these cells following the experimental design schematically depicted in Supplementary Fig. 1A. Human M∅s were pre-co-cultured with two different tumor cell lines, a human epidermal carcinoma (A431) and a human cervical tumor (HeLa), for 6 h. After this time, CD14 positive cells (CD14+) were isolated

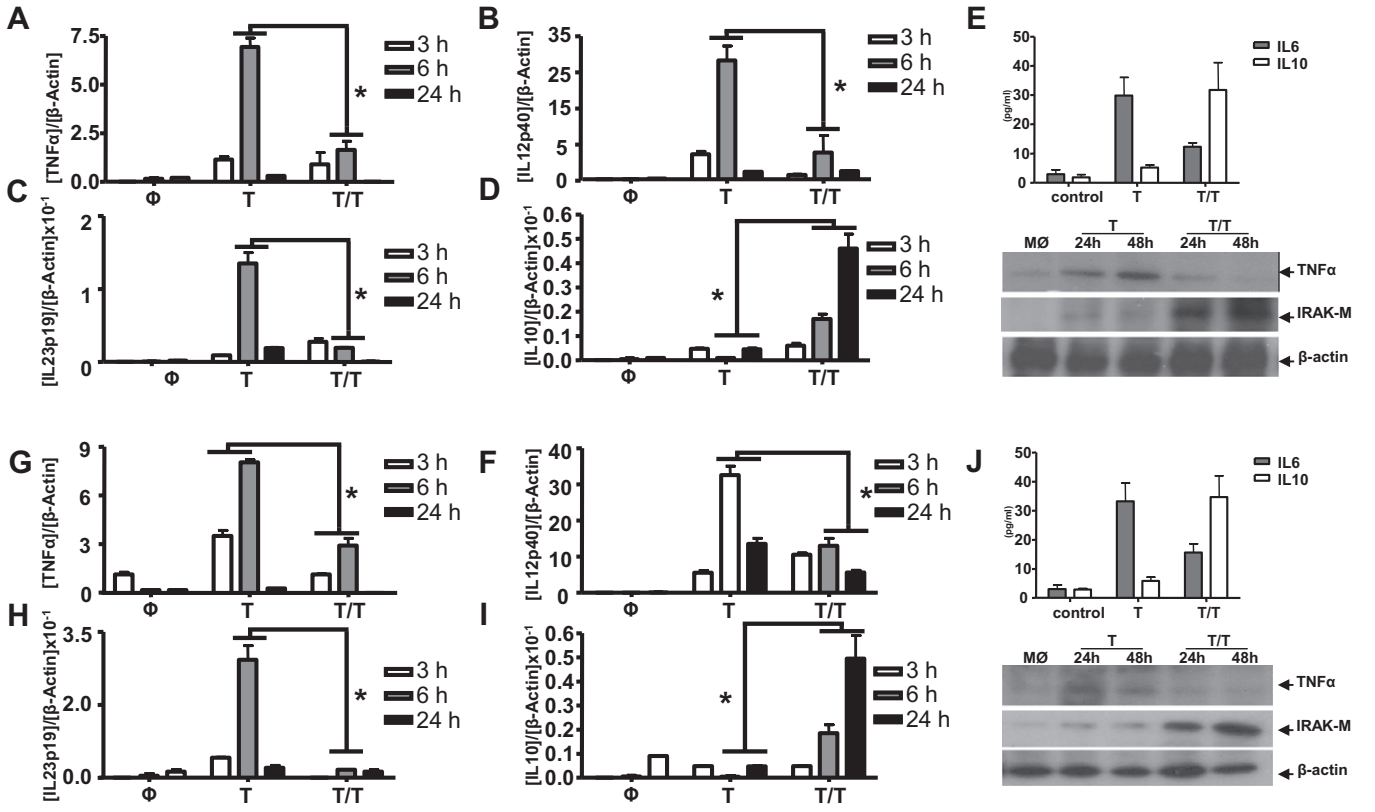

Fig. 1. Tumor cells induced a downregulation of inflammation in human monocytes/macrophages. M∅s were co-cultured once (T) or twice (T/T), ts = 3, 6, 24 and 48 h, with A431 (A–E) or HeLa (G–J) following the model showed in Supplementary Fig. 1A. Next, mRNA expression of several cytokines was analyzed in isolated CD14 + cells by real time Q-PCR (A–D) and (G–I). The ratio [gene]/[β-actin] is given ($n = 4$). $^*p < 0.01$ T versus T/T. (E and J, *upper panel*). Protein levels of IL6 and IL10 in the supernatant of cultures were analyzed by ELISA (ts = 24 h, $n = 4$). (E and J, *lower panel*) Intracellular expression of TNFα and IRAK-M was studied by Western Blot; a standard result is showed ($n = 3$).

and exposed once again to the same tumor cell line, for a time denominated "ts" (time of stimulation). Next, the CD14 + cells were isolated and analyzed. As we further demonstrated, this experimental condition reproduces tumor-tolerant M∅s that we shall name TT. As a control we used a single exposure of human M∅s to the tumor cell lines (condition: T). In all cases, the CD14 marker was analyzed to confirm the purity of our cells (Supplementary Fig. 1B). Note that CD14 + isolated M∅s (T/T) were cultured for at least 14 days and, in contrast to tumor cells, no proliferation was observed (Supplementary Fig. 1C). In addition, these cells were injected in nude mice to confirm the absence of tumor cell contamination. After two months no tumor growth was observed in mice that were injected with CD14 + isolated monocytes (T/T) (data not shown).

Our data indicate that pre-co-culture with a cancer cell line induces a refractory state to further tumor challenges. While the first encounter with both A431 and HeLa provoked high levels of TNFα, IL12p40 and IL23p19 mRNA, a second exposition to these cells did not induce the same inflammatory response (Fig. 1 A–C for A431 and G–H for HeLa). In strict contrast, the anti-inflammatory cytokine IL10 was up regulated during the second challenge with the tumor cells (Fig. 2 D and I). The protein levels of extracellular IL6 and IL10, and intracellular TNFα were analysed by ELISA and Western blot analysis (Fig. 1 E and J). We have also confirmed the up regulation of the negative regulator of inflammation, IRAK-M, in TT versus T. (Fig. 1 E and J). Note that neither contact with homolog lymphocytes nor fibroblasts induced tumor tolerance in M∅s (Supplementary Fig. 2). In summary, here our studied tumor-tolerant M∅s exhibited an M2-like signature phenotype [18,30].

3.2. Low MHC class II expression on tolerant monocytes/macrophages

In previous studies we have described LPS-tolerant M∅s failing to mount a standard inflammatory response against a second endotoxin-challenged while exhibiting a significant down-regulation of several members of MHCII [31]. Others and we have demonstrated that tumor-tolerant M∅s show an analogous profile of cytokines expression similar to LPS-tolerant ones [2,16]. In addition, in both contexts: tumor and sepsis, the IRAK-M expression plays a crucial role [2,10,23,32,33]. Taking into consideration previous results, we hypothesize that tumor-tolerant M∅s, as well as LPS-tolerant cells; also exhibit an impaired antigen presentation. To study this feature, we analyzed the protein expression of HLA-DQ and HLA-DR by FACS. Data presented in Fig. 2 (A-B, for A431 and E-F, for HeLa) undoubtedly indicate that these two factors are down regulated in tumor tolerant M∅s as was also observed in LPS-tolerant cells [31]. These findings prompted us to study the master regulator of MHC class II gene expression, CIITA (Class II Trans-Activator) [34–36]. This factor is down regulated in tumor tolerant M∅s (Fig. 2 C and G). Eventually, we verified the functional impact of CIITA/MHC-II down regulation via a standard lymphocyte proliferation assay [27,28]. In full agreement with the findings presented above, antigen presentation could be significantly impaired in tumor tolerant M∅s (Fig. 2 D and H).

3.3. Phagocytosis is enhanced in tolerant human monocytes/macrophages

Our data indicate that tumor-tolerant human M∅s, like in LPS-tolerance scenery; shown a patent M2-like phenotype as we have

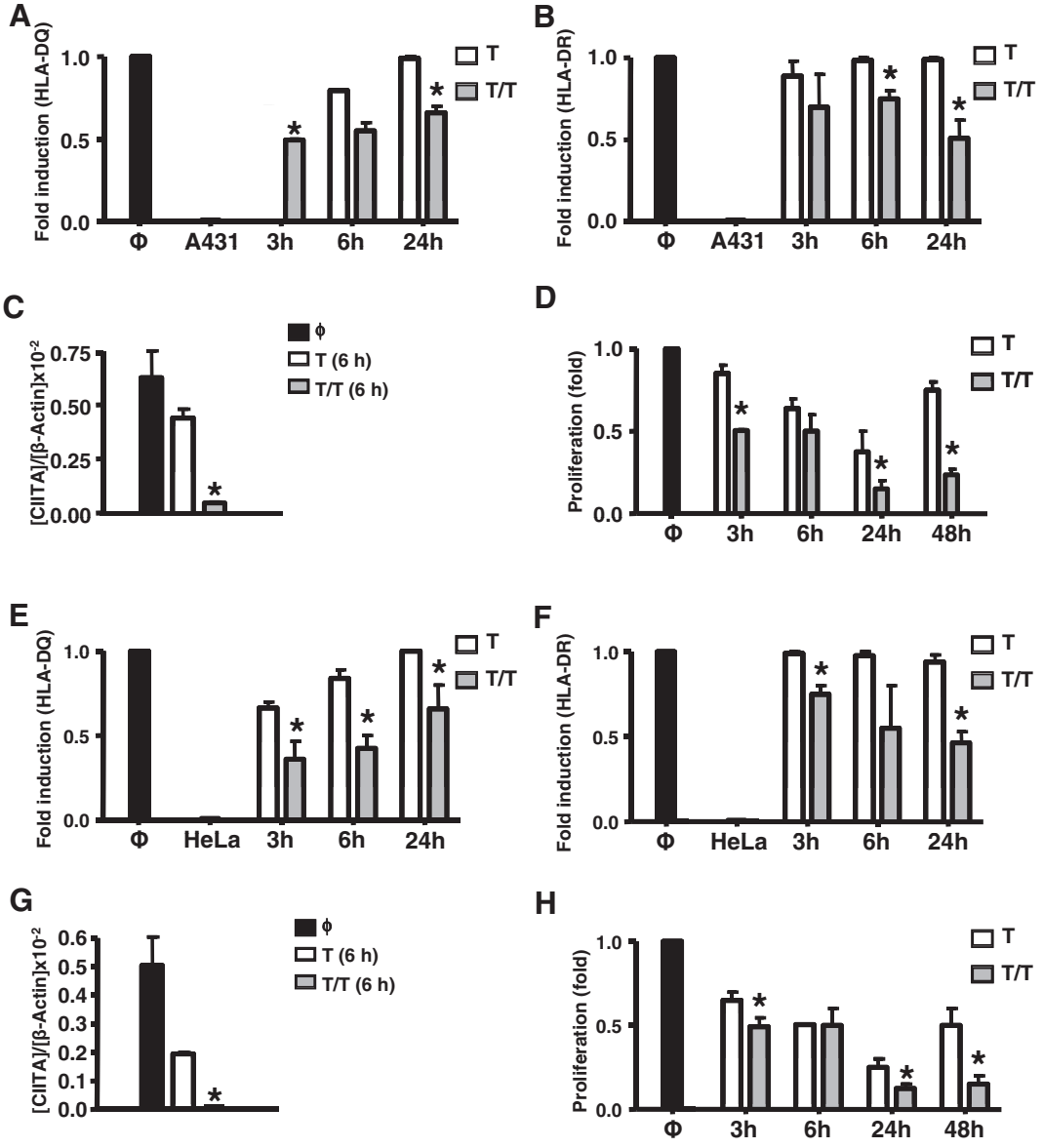

Fig. 2. MHC class II molecules and the MHCII master regulator CIITA are down regulated in tumor-tolerant monocytes/macrophages. M∅s were co-cultured once (T) or twice (TT) with the indicated tumor cells, ts = 3, 6 and 24 h, following the model showed in Supplementary Fig. 1A. (A and B, E and F) Isolated CD14 + monocytes were tested for the cell surface expression of HLA-DQ (A and E) and HLA-DR (B and F) by flow cytometry ($n = 4$). $^{*}p < 0.05$ T versus T/T. The *CIITA* was also analyzed in monocytes under (T) or (T/T) conditions, ts = 6 h (C and G), $^{*}p < 0.05$ T versus T/T ($n = 4$). (D and H) The same cells (T and T/T) and control monocytes (∅, solid bars) were exposed to human heterologous lymphocytes, a non-adherent cell population in the protocol used to obtain monocytes, labeled with the membrane stain PKH2 Green Fluorescent Cell Linker Kit. Next, lymphocyte proliferation was measured as loss of green fluorescence intensity in the CD3 + gate; in this analysis, cultures of stained lymphocytes not exposed to monocytes were used as control. The fold induction is shown ($n = 4$). $^{*}p < 0.05$ T versus T/T.

corroborated when analyzing the cytokine expression profile, the MHCII levels and the lymphocyte proliferation induction. In a previous work we have demonstrated that LPS-tolerant M∅s fail to produce antigen presentation when exhibiting an enhanced bacterial phagocytic activity [31]. In our present model we have also corroborated this enhanced phagocytic ability of tumor-tolerant M∅s by a standard phagocytosis assay of GFP-labeled bacteria (Fig. 3 A and B). These data establish that enhanced phagocytic activity is a physiologically relevant feature of tumor-tolerant M∅s.

3.4. Circulating monocytes from Chronic Lymphocytic Leukemia patients exhibit a tumor tolerant phenotype

To determine the relevance of our results *in vivo*, we analyzed the innate immune response of circulating monocytes isolated from fifteen non-treated CLL patients co-cultured with tumor cell lines. These patients were classified as CLL in accordance with the fourth edition of the WHO Classification of Tumors of Hematopoietic and Lymphoid Tissues. Due to CLL circulating monocytes being in contact with tumor cells [20], we hypothesized that these monocytes must be "locked" into a tumor-tolerant state and that a new contact with tumor cells would develop the features described above. As shown in Fig. 4, we corroborated the tolerant phenotype in the CLL patients' cohort. After co-culture with A431 and HeLa, CLL circulating monocytes expressed low levels of IL6 (Fig. 4A), increased their IL10 secretion (Fig. 4B) and reduced the cell surface's expression of both, HLA-DQ and HLA-DR (Fig. 4 C and D). In addition, these monocytes failed to induce lymphocyte proliferation in a standard proliferation assay (Fig. 4E) and exhibited a high phagocytic activity in presence of GFP-modified bacteria (Fig. 4F).

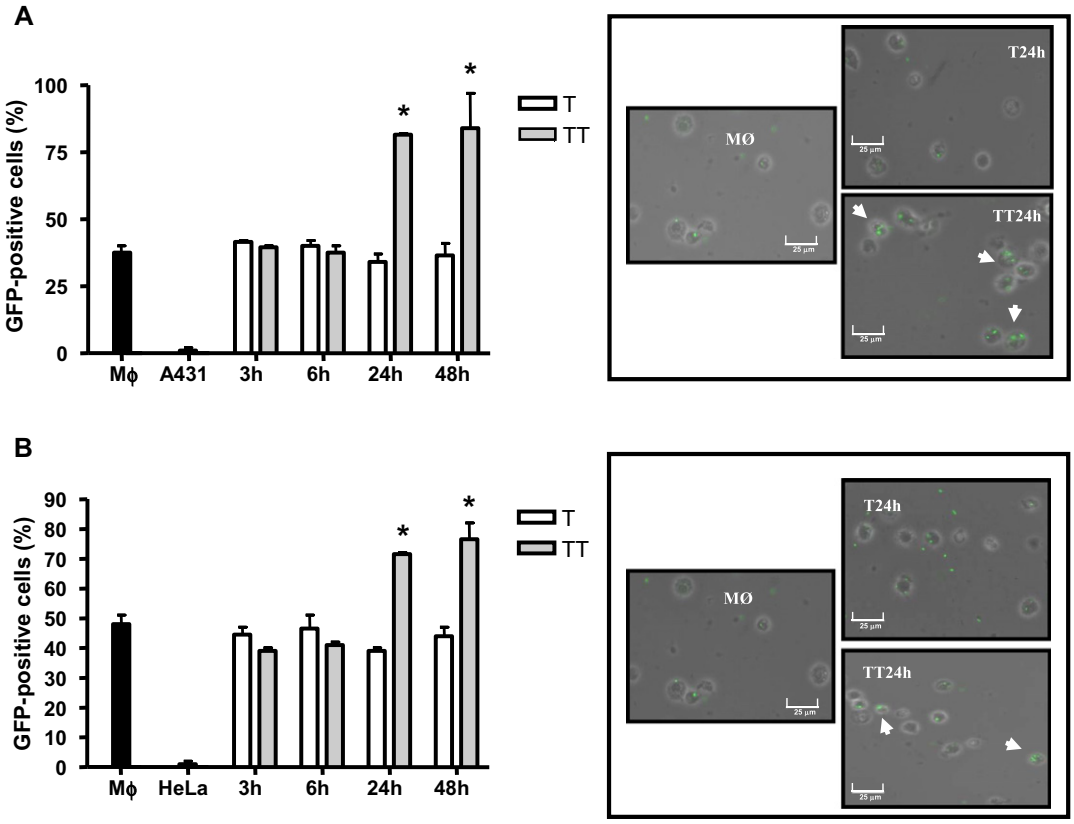

Fig. 3. Tumor-tolerant monocytes/macrophages exhibit high phagocytic ability. M∅s were co-cultured once (T) or twice (T/T) with the indicated tumor cells (A, A431 and B, HeLa), ts = 3, 6, 24 and 48 h, following the model showed in Supplementary Fig. 1A. Then, CD14 + monocyte were exposed to *E. coli* DH5-bacteria expressing isopropyl-d-thiogalactoside-inducible GFP (10^8 bacteria/ml) for 1 h. Adherent cells were harvested and analyzed by fluorescent microscopy (some monocyte internalized bacteria are indicated with arrows, right panels) and by flow cytometry (left panels); the percentage of green-positive monocytes is given ($n = 3$), $^*p < 0.05$ T versus TT.

4. Discussion

Since M∅s have the potential to express pro and anti-tumor activity, these cells are considered a double-edged sword in cancer development [37]. Several previous authors have demonstrated that when these cells are in contact with a tumor, first, they activate a rapid inflammatory response [8,38,39] and second, they reprogram their activity and become tolerant to the tumor [8,39]. In this regard, tumor-tolerant M∅s orchestrate various aspects of cancer, including matrix deposition and remodeling, construction of a metastatic niche, amongst other features, and exhibit an M2-like properties [16], characterized by a down-regulation in the expression of several cytokines.

We established a model to study tumor tolerance in human M∅s. In addition to the down regulation of the main proinflammatory cytokines and the up regulation of IL10 in tumor-tolerant M∅s, we also observed a significant over expression of the TLRs pathway's negative regulator: IRAK-M. As we have previously described, the presence of this pseudokinase could be crucial for inflammation control in both cancer and sepsis [2,10,23,29,32]. Besides over expression of inflammation's negative regulator and, its subsequent decrease of several cytokines, we also observed a patent repression of several factors involved in the antigen presentation of tumor-tolerant monocytes. Here we show that, like in LPS-tolerant monocytes, the master regulator of MHC class II genes (CIITA) are significantly down regulated in tumor tolerant cells. This finding suggests that reduced levels of CIITA could cause MHC class II deregulation during tumor-tolerance. Its precise mechanism is still unknown but could involve an epigenetic change of histones associated with CIITA.

Taking into account all these data, we hypothesized that antigen presentation would be diminished in tolerant human M∅s. This was later confirmed with a standard alloimmune lymphocyte proliferation assay. While naive human M∅s challenged with A433 and HeLa were able to induce proliferation of heterologous lymphocytes, tumor-tolerant M∅s re-co-cultured with the same tumor exhibiting a significantly lower stimulation of lymphocyte proliferation. Evidently, the observed failure in Ag presentation inhibits an appropriate switch to an adaptive immune response. Thus, a fine balance between refractory and inflammatory states appears to be a compromise solution in clinical situations. Furthermore our tumor-tolerant M∅s exhibited a high phagocytic ability when exposed to GPF-bacteria. Not unlike endotoxin-tolerant monocytes [31], these cells enhance their phagocytic capacity in a tumor environment.

Collectively our results indicate that tumor-tolerant M∅s exhibit a reduced inflammatory response and an increased phagocytic ability, but nevertheless their antigen presentation functions are impaired. These data suggest a high similarity between tumor- and endotoxin-tolerant M∅s' phenotype [10,31].

To explore the pathophysiological implications of human M∅s' tolerant phenotype, we decided to study the status of circulating monocytes of patients who suffer from Chronic Lymphocytic Leukemia. Since cancer cells in these patients are detected in circulation, their peripheral M∅s are in permanent contact with tumor cells. In this regard, the first contact between M∅s and tumor cells took place *in vivo* and we studied their response after a second contact with tumor cells *ex vivo*. Our results indicated that CLL M∅s showed the main features observed in our model. In contrast to cells from healthy controls, CLL M∅s did not develop a proper

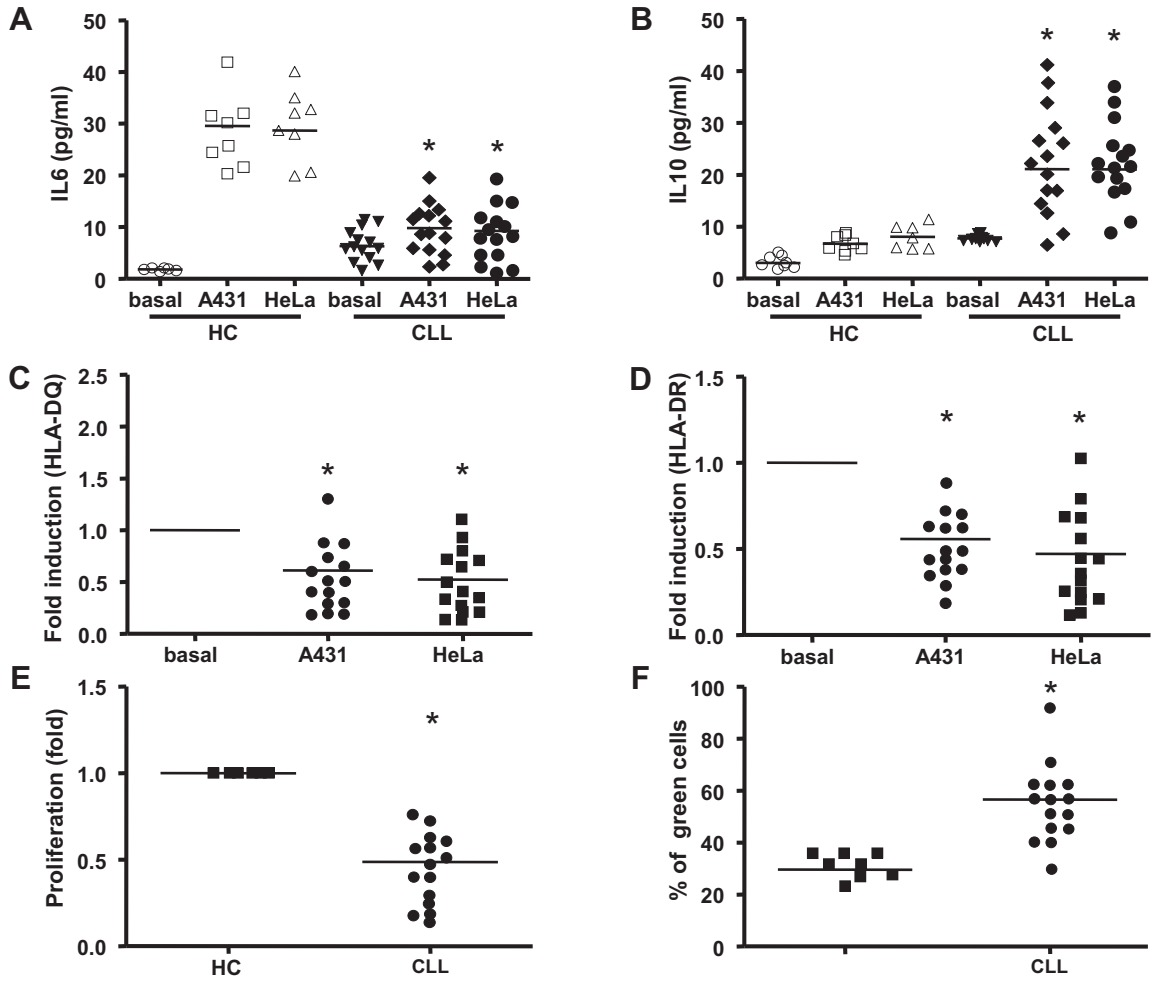

Fig. 4. Circulating monocytes from patients who suffer from Chronic Lymphocytic Leukemia exhibit a tumor tolerant phenotype. (A and B) Monocytes isolated from healthy controls (HC, $n = 8$) and Chronic Lymphocytic Leukemia (CLL, $n = 15$) were isolated and exposed to the indicated tumor cells for 24 h. Then, soluble levels of IL6 (A) and IL10 (B) were analyzed by ELISA. $^{*}p < 0.05$, CLL versus HC. (C and D) Monocytes isolated from CCL patients (CLL, $n = 15$) were exposed to the indicated tumor cells for 24 h, next CD14 + adherent cells were stained for HLA-DQ (C) and HLA-DR (D) and the expression of these cell surface molecules were analyzed by flow cytometric. Fold induction with respect to their basal is shown, $^{*}p < 0.05$, tumor-treatment versus basal. (E) Monocytes from HC and CLL patients were exposed to human heterologous lymphocytes following the same protocol described in Fig. 2D. The proliferation fold induction is shown, $^{*}p < 0.05$, CLL versus HC. (F) Monocytes from HC and CLL patients were exposed to *E. coli* DH5-bacteria expressing isopropyl-d-thiogalactoside-inducible GFP following the same protocol described in Fig. 3, $^{*}p < 0.05$, CLL versus HC.

inflammatory response, thus failing to induce lymphocyte proliferation and exhibit high phagocytosis ability.

In summary, our current results indicate that tumor-tolerant monocytes share the main features with LPS-tolerant cells in humans namely, high phagocytic activity but low Ag presentation ability and a down regulation of inflammatory response. Additionally, we showed that the phenotype of CLL M∅s mirrors the tumor tolerance state in a pathological setting. These findings validate the data obtained with our *in vitro* model system, and they provide a characterization of CLL circulating M∅s. Besides and given the increasing interest in exploiting human cells' tolerant behavior as a therapeutic target, our results may need to be considered in several clinical situations.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbrc.2012.05.124.

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