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Tumor Cells Deactivate Human Monocytes by Up-Regulating IL-1 Receptor Associated Kinase-M Expression via CD44 and TLR4[1]

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Although blood monocytes possess significant cytotoxic activity against tumor cells, tumor-infiltrating monocytes are commonly deactivated in cancer patients. Monocytes pre-exposed to tumor cells show significantly decreased expression levels of TNF-α, IL-12p40, and IL-1R-associated kinase (IRAK)-1. Activation of the Ser/Thr kinase IRAK-1 is an important event in several inflammatory processes. By contrast, another IRAK family member, IRAK-M, negatively regulates this pathway, and is up-regulated in cultures of endotoxin-tolerant monocytes and in monocytes from septic patients within the timeframe of tolerance. In this study, we show that IRAK-M expression is enhanced at the mRNA and protein level in human monocytes cultured in the presence of tumor cells. IRAK-M was induced in monocytes upon coculturing with different tumor cells, as well as by fixed tumor cells and medium supplemented with the supernatant from tumor cell cultures. Moreover, blood monocytes from patients with chronic myeloid leukemia and patients with metastasis also overexpressed IRAK-M. Low concentrations of hyaluronan, a cell surface glycosaminoglycan released by tumor cells, also up-regulated IRAK-M. The induction of IRAK-M by hyaluronan and tumor cells was abolished by incubation with anti-CD44 or anti-TLR4 blocking Abs. Furthermore, down-regulation of IRAK-M expression by small interfering RNAs specific for IRAK-M reinstates both TNF-α mRNA expression and protein production in human monocytes re-exposed to a tumor cell line. Altogether, our findings indicate that deactivation of human monocytes in the presence of tumor cells involves IRAK-M up-regulation, and this effect appears to be mediated by hyaluronan through the engagement of CD44 and TLR4. ***The Journal of Immunology,*** **2005, 174: 3032–3040.**

Monocytes/macrophages are involved in the immune response to tumors as they present tumor-associated Ags and act as cytotoxic effector cells. There is considerable evidence that cancer cells induce the expression of proinflammatory cytokines and reactive oxygen species in monocytes. In addition, it has been shown that CD44, the major receptor for hyaluronan (HA)[4], is involved in the signaling cascade responsible for their generation (1–8). HA is a glycosaminoglycan involved in the maintenance of matrix structure and other cellular functions. Moreover, HA is produced by many tumor cells (7), and its overexpression correlates with the tumor metastatic potential (8).

The protective influence of tumor-infiltrating macrophages (TIMs) is depressed in cancer patients (9, 10), as tumors counteract the cytotoxic and proinflammatory activities of TIMs in their local environments (11, 12). Neutralization of TIMs involves multiple mechanisms, among them the production of certain cytokines and prostaglandins by tumor cells (5, 6). This in turn leads to the down-regulation of TNF-α, IFN-γ, IL-12p40, and other cytotoxic and proinflammatory factors produced by monocytes (7, 9, 11, 12). These processes reflect the need for cancer cells to develop mechanisms that inhibit inflammation (6, 13). Indeed, tumor cells induce a specific kind of tolerance in cultures of human monocytes, and this unresponsiveness state involves the down-regulation of TNF-α, IL-12p40, and IL-1R-associated kinase (IRAK)-1 expression, as well as the subsequent inhibition of their antitumor activity (see Ref. 12). In the latter study, it was shown that monocyte deactivation involves the generation of HA by tumor cells and its recognition by CD44 on monocytes (12).

During tumor development, inflammation may be triggered by receptors recognizing nonself molecules on tumor cells. The family of TLRs is an important mediator of the innate response (14–16), and activation of these receptors triggers the production of several molecules involved in antitumoral responses such as IFNs (17). Following recognition of nonself molecules, the receptors bind intracellular adaptor proteins, such as MyD88 and TIR domain-containing adaptor protein/Mal, which in turn recruit other proteins implicated in inflammation signaling (e.g., IRAK-1, IRAK-4, and TNFR-associated factor 6; see Refs. 16, and 18–25). The resulting multiprotein complex seems to play an important

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[4] Abbreviations used in this paper: HA, hyaluronan; IRAK, IL-1R-associated kinase; Q-PCR, quantitative PCR; TIM, tumor-infiltrating monocytes; siRNA, small interfering RNA.

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role in the intracellular signaling elicited upon TLR activation, apparently through activation of MAPK and NF-κB pathways (16, 18, 20, 21, 25–29). The IRAK family of kinases is essential in this pathway, recently illustrated by the discovery of a polymorphism in IRAK-4 that induces a series of infectious episodes in patients (30). Similarly, it appears that phosphorylation of IRAK-1 is essential in the transduction of the inflammatory signal (31). In human monocytes, IRAK-1 is down-regulated when, following exposure to a tumor cell, monocytes are cocultured with either the same or different tumor cells (12). This down-regulation mechanism disrupts the inflammatory response against cancer progression.

Another member of the IRAK family, IRAK-M, is also involved in endotoxin tolerance in monocytes (32–34). This nonfunctional kinase appears to be exclusively expressed in monocytes/macrophages, and has a negative rather than a positive influence on the signaling pathways activated by the other family members (32–35). In fact, it appears that IRAK-M prevents dissociation of the activator complex formed upon TLR4 engagement (35). We have previously shown that human monocytes rapidly up-regulate IRAK-M expression after LPS stimulation during endotoxin tolerance, as confirmed in monocytes from septic patients that were within the tolerance timeframe (33). We also found that IRAK-M expression is not restricted to the TLR pathway, as human monocytes treated with a NO donor expressed large amounts of this protein, apparently under the control of TNF-α (36).

On the basis of these observations, we hypothesized that IRAK-M could be involved in the deactivation of TIMs mediated by tumor cells. We have now tested this hypothesis in a human model of TIMs. We also studied the role of IRAK-M in our system by blocking IRAK-M expression through specific small interfering RNAs (siRNAs). Finally, we examined whether HA, its receptor CD44, TLR4, or TLR2 could influence the activation of IRAK-M in this model, and analyzed IRAK-M expression in blood monocytes from patients with chronic myeloid leukemia or metastatic tumors.

Materials and Methods

Reagents

All reagents were of the highest quality available and were obtained from Merck, Boehringer, or Sigma-Aldrich. LPS (from *Salmonella abortus*) was generously provided by Dr. Chris Galanos from the Max-Planck-Institut für Immunobiologie, Freiburg, Germany. The medium used for cell culture was DMEM from Invitrogen Life Technologies. The rabbit anti-IRAK-M polyclonal antiserum was obtained from Chemicon International. Mouse anti-β-actin anti-TLR4, anti-TLR2, and rabbit anti-TNF-α mAbs were purchased from Santa Cruz Biotechnology and anti-CD44 was from AL Immunotools. CD-14-FITC was from Cymbus Biotechnology. The LS Separation Columns system and the anti-CD14 magnetic beads were from Miltenyi Biotech.

Patient selection

Data included in this study were obtained from six patients (age 29 ± 4 years, mean ± SD) consecutively admitted to the Department of Hematology at the Hospital La Paz (Madrid, Spain) with chronic myeloid leukemia as confirmed by BCR/ABL test, and from two patients (age 56 ± 5 years) admitted to the Department of Internal Medicine at the Hospital La Paz with untreated disseminated gastric carcinoma with metastasis in the peritoneum, liver, blood, and lung. Two patients with localized colon tumors admitted to the Department of Oncology were also studied (age 58 ± 2 years). The following exclusion criteria were imposed: treatment with immunosuppressive drugs in the month before obtaining the sample, presence of HIV or hepatitis B/C virus, pregnancy, and age above 70 years. Written informed consent was obtained from all subjects and the study was approved by the local ethics committee. The control group consisted of 10 healthy volunteers (age 40 ± 12 years). Blood samples were taken from patients and healthy donors, from which $CD14^+$ monocytes were isolated. Total monocyte RNA was isolated and used to synthesize cDNA.

PMBC isolation and cell culture

PBMCs were isolated from the blood of healthy donors by centrifugation on Ficoll-Hypaque Plus (Amersham Biosciences). Cells were initially cultured for 2 h to a density of 10^6 cells/ml in DMEM supplemented with antibiotics (100 IU/ml penicillin and 100 μg/ml streptomycin). After this period, the supernatant was removed and adherent cells were cultured in the same medium additionally supplemented with 10% heat-inactivated normal serum pooled from healthy volunteers and inactivated for 16 h. The cells were then either 1) cocultured with different tumor cell lines, fixed or alive, or 2) cultured in the presence of HA or medium containing 20% of the supernatant from tumor cell lines cultured for 24 h, for the periods indicated, or 3) used for transfections. After each treatment, human monocytes were isolated using the LS-positive Separation Column System (Miltenyi Biotech) with an anti-CD14 Ab conjugated to magnetic beads following the manufacturer's recommendations. In some experiments, cultures were additionally treated with blocking Abs against CD44, TLR2, and/or TLR4 at the concentrations indicated. HA solutions were tested for LPS contamination immediately before they were used in indicated experiments.

$CD14^+$ monocytes from patients were isolated by centrifugation on Ficoll-Hypaque Plus (Amersham Biosciences) followed by recovery on LS-positive Separation Columns using an anti-CD14 Ab conjugated to magnetic beads.

Cell lines and primary cultures of human fibroblasts and lymphocytes

The following human cell lines were used: A431 (human epidermal carcinoma), MCF7 (human breast carcinoma cell line), HL60 (human promyelocytic leukemia cell line), Jurkat (human T cell leukemia), HepG2 (human hepatoblastoma cell line), and HeLa (human cervical tumor) provided by the American Type Culture. All cell lines were tested for mycoplasma and LPS contamination. Primary cultures of human fibroblasts were obtained as previously reported by Janssens et al. (35). Primary cultures of human lymphocytes were obtained by centrifugation of blood from healthy volunteers on Ficoll-Hypaque Plus (Amersham Biosciences) and then culturing the cells for 16 h in complete DMEM, after which nonadherent cells were considered as lymphocytes. Cells were fixed with paraformaldehyde following a standard protocol.

Cocultures and cell isolation

Human monocytes were cocultured with different tumor cell lines at a ratio of 1:0.3 (monocytes:tumor cells) for the times indicated. After incubation, pooled cells were harvested and stained with an anti-CD14 Ab conjugated to magnetic beads. CD14-positive cells ($CD14^+$) were isolated using the LS Separation Column System and recovered for further assays. The purity of sorted cells was analyzed by flow cytometry and was >92%. Isolated $CD14^+$ monocytes were used for RNA extraction, protein isolation, culturing in the presence of differing concentrations of HA, or re-exposure to tumor cell lines or control cells. An identical isolation procedure was conducted after re-exposure of monocytes to tumor cell lines or control cells.

RNA and protein isolation

After treatment, cells were washed once with PBS and their RNA was isolated using TRI-Reagent (IMICO). The purified RNA was treated with RNase-free DNase I (Amersham Biosciences), and cDNA was obtained by reverse transcription of 1 μg of RNA using a poly(dT) oligonucleotide primer (Roche Diagnostics). Protein fractions were isolated as recommended (Roche Diagnostics), and the final pellets were dissolved in a solution containing 1% SDS.

mRNA quantification

The expression levels of IRAK-M, TNF-α, IL-12p40, and 18S were analyzed by real-time quantitative PCR (Q-PCR) (LightCycler; Roche Diagnostics), using cDNA obtained as described above. Real-time PCR was performed using a Fast-Start DNA master SYBR Green system (Roche Diagnostics) and specific primers (listed below). All results were normalized to the expression of the 18S gene, and the cDNA copy number of each gene of interest was determined using a seven-point standard curve. Standard curves were run with each set of samples, the correlation coefficients r^2 for the standard curves being >0.99. To confirm the specificity of the reaction products in each experiment, the melting profile of each sample was analyzed using the LightCycler. The melting profile was determined by maintaining the reaction at 80°C for 10 s and then heating it to 95°C at a linear rate of 0.1°C/s while measuring the emitted fluorescence. An analysis of the melting curves demonstrated that each pair of primers amplified a single product. PCR products were separated in agarose gels and stained with ethidium bromide (0.5 μg/ml) to verify amplification of a single fragment of the predicted size. Each LightCycler PCR run consisted of 45 cycles with an initial denaturation of 5 min at 95°C. For TNF-α the cycles

used were: 95°C for 10 s, 64°C for 10 s, and 72°C for 10 s; for 18S: 95°C for 10 s, 60°C for 10 s, and 72°C for 10 s; for IRAK-M: 95°C for 10 s, 59°C for 10 s, and 72°C for 19 s; and for IL-12p40: 95°C for 10 s, 64°C for 10 s, and 72°C for 10 s.

Primers

The sequences of oligonucleotides used are: IRAK-M: sense 5′-TTT GAA TGC AGC CAG TCT GA-3′, antisense 5′-GCA TTG CTT ATG GAG CCA AT-3′; TNF-α: sense 5′-GCC TCT TCT CCT TCC TGA TCG T-3′, antisense 5′-CTC GGC AAA GTC GAG ATA GTC G-3′; and IL-12p40: sense 5′-GAC ATT CAG TGT CAA AGC AGC A- 3′, antisense 5′-CCT TGT TGT CCC CTC TGA CTC T-3′.

For 18S mRNA detection we used the primers of QuantumRNA Classic 18S provided by Ambion. All other primers were synthesized, desalted, and purified by IZASA.

ELISA for TNF-α

The levels of soluble TNF-α were measured using an immunoassay kit purchased from CLB.

Western blot analysis

Cell extracts were denatured by boiling in Laemmli buffer, resolved on 12% SDS-polyacrylamide gels in Tris/glycine/SDS buffer (25 mM Tris, 250 mM glycine, and 0.1% SDS), and transferred to Immun-Blot PVDF Membranes (Bio-Rad) at 300 mA for 1.5 h at 4°C. After blocking for 1 h in 20 mM Tris-HCl, 150 mM NaCl, 0.2% Tween 20 (TBST) containing 5% nonfat milk, membranes were washed three times in TBST alone and probed for 20 h with Abs diluted in TBST. Following washing in TBST, the membranes were incubated with a secondary HRP-conjugate (diluted 1/2000) for 30 min and washed three times in TBST. The bound Abs were detected using ECL Plus reagents according to the manufacturer's instructions (Amersham Pharmacia Biotech).

Apoptosis assay

After treatment, cells were washed and resuspended in PBS. The apoptosis assay was performed using an AnnexinV-FITC/PI Apoptosis Detection kit (Oncogene) according to the manufacturer's recommendations. The stained cells were analyzed in a BD FACSCalibur flow cytometer (BD Biosciences) equipped with a 25-mW argon laser. The proportion of apoptotic cells was quantified by plotting the Annexin V-FITC against the PI fluorescence.

Design and transfection of siRNAs

Design of specific siRNA molecules was performed by Ambion. The oligonucleotide sequences used were as follows: sense, 5′ GGA GAU GGG ACA UCG UCG Att 3′; and antisense, 5′ UCG ACG AUG UCC CAU CUC Ctg 3′.

These oligonucleotides were synthesized by and purchased from Ambion. In vitro synthesized siRNAs or control RNA were transfected into human monocytes using a Silencer siRNA Transfection Kit (Ambion) following recommendations of the manufacturer. We used as carrier siPORT Amine produced for Ambion by Mirus Corp. Cells were incubated in antibiotics-free 3% FBS for 20 h posttransfection before performing further investigations.

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.

Results

Tumor cells induce deactivation of human monocytes in vitro

To study the mechanisms involved in the deactivation of human monocytes by tumor cells, we first set out to establish conditions under which this process can be reliably reproduced in vitro. We tested the homogeneity of our human monocyte cultures by analyzing the expression of the specific surface marker CD14 by flow cytometry (CD14-FITC). On average, 92% of the cells in culture expressed this marker (data not shown). Next, these cells were cultured alone or together with different human tumor cell lines for 6 h before harvesting and collecting the $CD14^+$ monocytes. These $CD14^+$ cells were then restimulated with the same cancer cells for up to 18 h.

The transcriptional levels of TNF-α and IL-12p40 were quantified 1 h and 3 h after stimulation, respectively, using real-time Q-PCR (LightCycler system). Although the transcription of both TNF-α and IL-12p40 was up-regulated when human $CD14^+$ monocytes were exposed to tumor cells lines for the first time, re-exposure to the same cancer cells induced a significant tolerance (Fig. 1, *A* and *B*). In contrast, neither fibroblasts nor lymphocytes were able to induce either TNF-α or IL-12p40 gene expression. Further, human monocytes pre-exposed to fibroblasts or lymphocytes did not become refractory to tumor cells (data not shown). In addition, TNF-α concentration in cell supernatants was determined after an 18-h stimulation using a commercially available ELISA. The levels of TNF-α were significantly diminished in monocyte supernatants pre-exposed to cancer cell lines (Fig. 1*C*), in accordance with published results (12).

IRAK-M is rapidly up-regulated in human monocytes pre-exposed to tumor cells

Having established a model to study the deactivation of human monocytes by cancer cells, we analyzed the expression of the *IRAK-M* gene in this system. We selected the A431 tumor cell line because these cells induced a representative response in human monocytes, and used human fibroblasts as control cells. The time

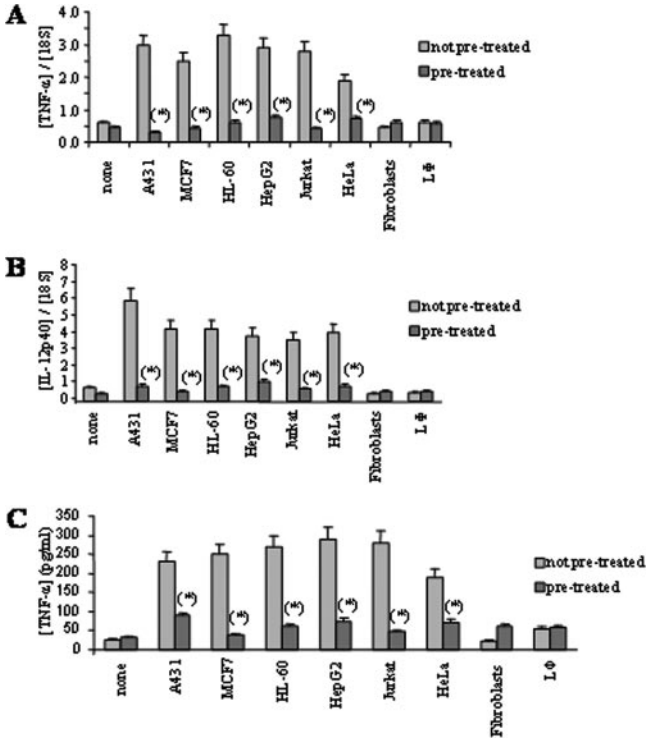

FIGURE 1. Tumor cells induce a tolerance state in human monocytes. Human monocytes were exposed to different tumor cells (filled bars) or maintained in culture medium alone (gray bars) for 6 h. $CD14^+$ monocytes were then recovered and restimulated with the same tumor cells they had encountered before. *A*, Cocultures of human monocytes were harvested 1 h after rechallenging, $CD14^+$ monocytes were recovered, and TNF-α/18S mRNA expression was quantified by real-time Q-PCR. *B*, Cocultures of human monocytes with the indicated tumor cell lines were harvested after 3 h coculture, $CD14^+$ monocytes were recovered, and IL-12p40/18S gene expression was analyzed by Q-PCR. *C*, Levels of TNF-α in the supernatants of these 18-h cocultures were determined by ELISA. All cocultures were conducted at a ratio of 1:0.3 (monocytes:tumor cells). The results shown are from a representative experiment ($n = 4$); ∗, $p < 0.05$ with respect to monocytes that were not pretreated with the indicated cells.

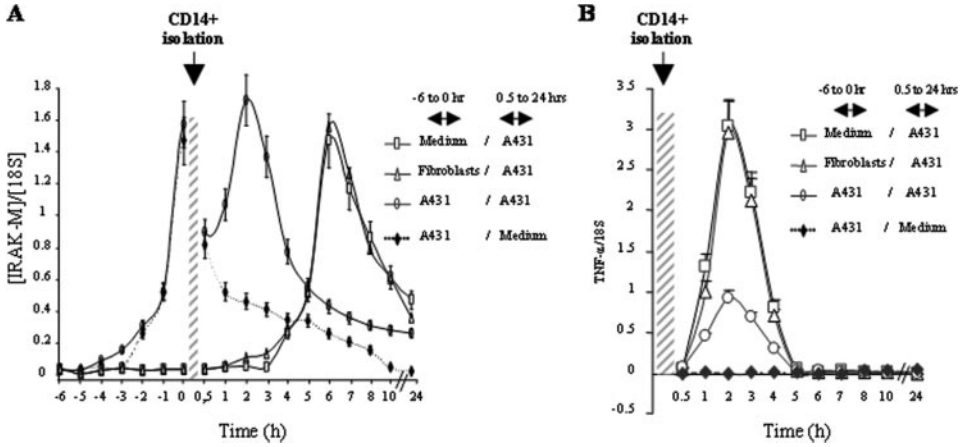

FIGURE 2. IRAK-M mRNA is expressed in human monocytes pretreated with A431 cells. Human monocytes were cultured for 6 h in the presence of A431 cells, fibroblasts, or medium alone. $CD14^+$ cells were then recovered and immediately restimulated with A431 or culture medium for the indicated times. These monocytes were recovered again and mRNA expression was determined. The ratios of IRAK-M/18S mRNA expression (*A*) and TNF-α/18S gene expression (*B*) were determined by real-time Q-PCR using a LightCycler system. In all cases, cocultures were performed at a ratio of 1:0.3 (monocytes:cells). The data shown correspond to a representative experiment ($n = 3$).

course of IRAK-M mRNA expression was studied in human monocytes pre-exposed to A431 cells or human fibroblasts, or cultured alone for 6 h, and then stimulated with the tumor cell line (Fig. 2*A*). As we showed previously, nonstimulated human monocytes did not express IRAK-M (33, 36). However, when cocultured with A431 cells for 6 h, *IRAK-M* was induced in the cells previously cultured alone or with fibroblasts. Moreover, a rapid up-regulation of IRAK-M expression was observed in those human monocytes pre-exposed to A431 cells and then exposed again to the same cancer cell line. Indeed, we observed a strong induction of IRAK-M mRNA in A431-pretreated human monocytes starting 2 h after re-exposure to the same tumor cells. This finding indicated that not only did A431 cells induce IRAK-M gene expression in human monocytes but also, that they preconditioned these monocytes to rapidly up-regulate IRAK-M expression in a second encounter with a tumor cell. In contrast, A431-pretreated human monocytes showed a 3-fold decrease in TNF-α mRNA expression after stimulation with the same tumor cells (Fig. 2*B*).

Tumor cells activate IRAK-M mRNA expression in human monocytes

We then analyzed whether IRAK-M gene expression was induced in human monocytes exposed to other cancer cell lines. In time course studies, all tumor cell lines tested induced a clear up-regulation of IRAK-M mRNA after 6 h in culture, which was comparable to or greater than that induced by LPS (10 ng/ml) used as a positive control. Interestingly, there were noticeable differences in both the kinetics and the maximum induction level of IRAK-M, with some cell lines inducing significant transcription 3 h after exposure (Fig. 3). In contrast, neither human fibroblasts nor lymphocytes expressed IRAK-M upon exposure to cancer cells.

Fixed tumor cell lines and soluble factors from cultured cancer cells induce IRAK-M gene expression in human monocytes

To gain more information as to how tumor cells might influence IRAK-M expression within the framework of tumor-induced monocyte deactivation, we studied the potential role of membrane-bound elements and of soluble factors derived from cancer cell lines on IRAK-M expression. All fixed tumor cells tested induced IRAK-M transcription in human monocytes 3 h after exposure, and this effect was maintained for up to 6 h. In contrast, no IRAK-M mRNA was detected when human monocytes were incubated with fixed human fibroblasts or lymphocytes within this time scale (Fig. 4*A*). These observations suggested that a membrane-bound molecule(s) from cancer cells was responsible for the induction of IRAK-M in human monocytes.

We also tested the possible role of soluble factors from tumor cell lines by culturing human monocytes in the presence of filtered medium conditioned by cancer cells for 24 h (20% supplement of the normal medium). Again, IRAK-M transcription was clearly up-regulated in human monocytes and could be detected after culturing for 3 h in the presence of the supplemented medium (Fig. 4*B*), whereas medium conditioned by human fibroblasts or lymphocytes had no effect on IRAK-M expression. Of note, the transcript levels were lower in human monocytes stimulated with conditioned medium than in those exposed to fixed cell lines.

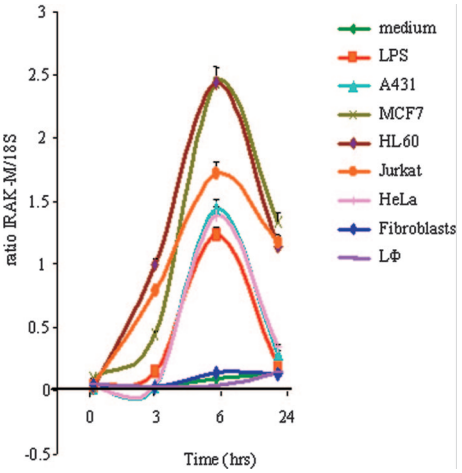

FIGURE 3. IRAK-M is overexpressed in human monocytes cocultured with different tumor cell lines. Human monocytes were exposed to tumor cells, human fibroblasts, human lymphocytes (ratio 1:0.3), or medium for the times indicated, and then the $CD14^+$ monocytes were recovered. The ratio IRAK-M/18S mRNA was determined by real-time Q-PCR. $CD14^+$ monocytes cultured in medium or stimulated with 10 ng/ml LPS were used as negative and positive controls, respectively. The results shown are from a representative assay ($n = 5$).

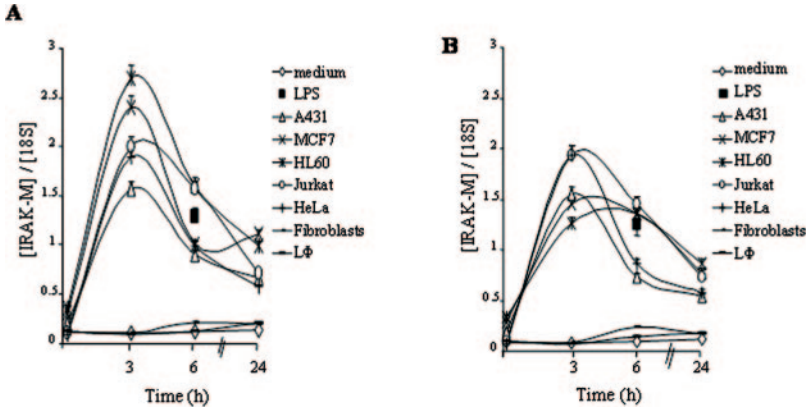

FIGURE 4. Both fixed tumor cells and soluble factors released from these cells induce IRAK-M expression in human monocytes. *A*, Cultures of human monocytes were incubated with fixed tumor cells (ratio 1:0.3) for the times given. At the indicated times, supernatants were removed, the cultures were washed twice with PBS and the cells were harvested. Quantitative analysis of IRAK-M/18S mRNA expression was conducted by real-time Q-PCR. *B*, Human monocytes were cultured in the presence or absence of medium supplemented with 20% filtered supernatant from tumor cell lines cultured for 24 h. At the indicated times, the medium was removed, the cultures were washed twice with PBS, and the cells were harvested. Q-PCR analyses were performed using the LightCycler system. In both cases, monocytes stimulated for 6 h with 10 ng/ml LPS were used as positive control. The data from a representative experiment are shown ($n = 4$).

HA induces IRAK-M mRNA expression

It has previously been shown that HA plays an important role in the induction of the inflammatory response in monocytes. The glycosaminoglycan is attached to the membranes of several cancer cells, but is also released by these cells as a soluble factor in culture (37–39). To study the possible role of HA in the up-regulation of IRAK-M by tumor cells, human monocytes were stimulated with two different concentrations of soluble HA. The transcription of IRAK-M was clearly up-regulated 3 h after cultures of human monocytes were supplemented with HA (Fig. 5*A*). In addition, HA did stimulate TNF-α transcription in human monocytes in a time-dependent manner (Fig. 5*B*).

IRAK-M protein accumulates in monocytes exposed to cancer cells or HA

To examine IRAK-M protein expression, we performed Western blot analysis with a specific antiserum against IRAK-M, using β–actin as a control for loading. IRAK-M protein expression was induced in human monocytes cocultured with fixed or living tumor cells (A431), as well as in those incubated in the presence of either A431 cell conditioned medium or HA (Fig. 6). We also found that all tested tumor cell lines induced IRAK-M protein expression in human monocytes after 6–8 h in coculture (data not shown).

Specific IRAK-M down-regulation reinstates TNF-α mRNA and protein expression in human monocytes re-exposed to cancer cells and HA

We next investigated whether experimental modulation of IRAK-M expression could alter cancer tolerance in human monocytes. As shown in Fig. 3, IRAK-M mRNA was clearly induced 6 h after first encounter with tumor cells, or upon stimulation with HA (Fig. 5*A*). IRAK-M was also induced 6 h after coculture with A431 or stimulation with HA in human monocytes transfected with control RNAs. In contrast, its expression was down-regulated in cells transfected with specific siRNAs for IRAK-M (Fig. 7*A*). The expression of IRAK-M mRNA in siRNA-transfected monocytes was <30% of the control experiment. Furthermore, a significant reduction in IRAK-M expression was detected in transfected cultures by Western blot analysis (data not shown).

Of particular note, TNF-α mRNA expression was significantly reinstated in siRNA-transfected monocytes after a second encounter with A431 cells, while monocytes transfected with control

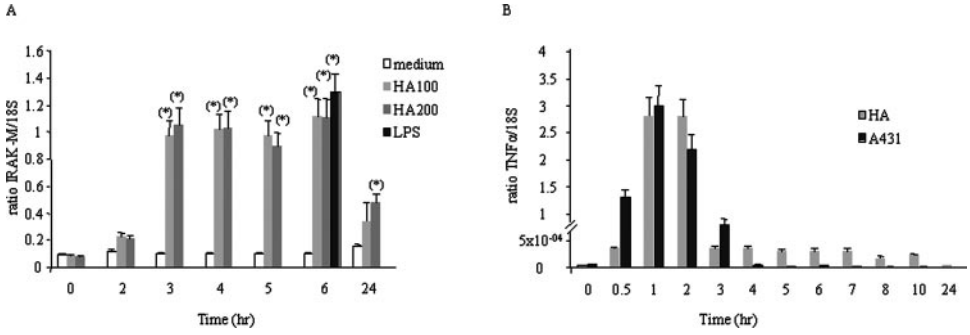

FIGURE 5. HA induces IRAK-M mRNA expression in human monocytes. *A*, Cultures of human monocytes were treated with 100 μg/ml (light gray bars) or 200 μg/ml HA (dark gray bars), for the times indicated. Then, the cultures were washed three times with PBS, the cells harvested and the total RNA was isolated. IRAK-M/18S gene expression was analyzed by real-time Q-PCR. Medium alone was used as a negative control (open bars) and a 6-h exposure to LPS (10 ng/ml) as a positive control (filled bars). A typical result is shown ($n = 4$); *, $p < 0.05$ with respect to nontreated cultures. *B*, Cultures of human monocytes were treated with 100 μg/ml HA (gray bars) or cocultured with A431 (filled bars) for the indicated times. $CD14^+$ cells were then recovered from the coculture and HA-stimulated cultures were washed twice with PBS. The ratio of TNF-α to 18S mRNA levels was assessed by real-time Q-PCR. Representative results are shown ($n = 3$).

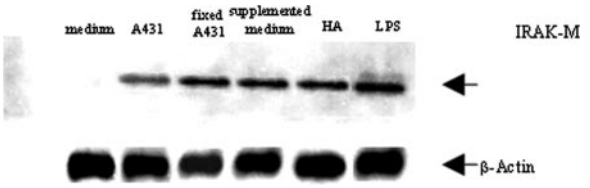

FIGURE 6. Accumulation of IRAK-M protein in tumor-treated monocytes. Human monocytes were either cocultured with A431 cells (ratio 1:0.3), stimulated with fixed A431 cells (ratio 1:0.3), cultured in the presence of medium supplemented with 20% filtered supernatant from cultures of A431, stimulated with 100 μg/ml HA, or treated with 10 ng/ml LPS for 24 h. The cultures were then washed three times with PBS and the cells were harvested. When cocultured, CD14$^+$ monocytes were recovered. Total protein was isolated and the expression of IRAK-M protein was analyzed by a Western blot using 20 μg of the total sample and an anti-IRAK-M polyclonal antiserum. An anti-β-actin mAb was used as a control of loading and transfer. The data from a representative experiment are shown ($n = 3$).

RNA were not able to express TNF-α, neither at mRNA nor protein levels upon re-exposure to this tumor cell line. Specificity of siRNA was confirmed by its observed dose-dependent action (Fig. 7*B*). In addition, TNF-α protein production was also reinstated in siRNA-transfected monocytes (Fig. 7*D*). Finally, a clear reinstatement of TNF-α mRNA expression was also observed in siRNA-transfected cultures restimulated with HA (Fig. 7*C*). These findings indicated that down-regulation of IRAK-M expression reverses the phenotype of cancer tolerance in human monocytes, and that HA could play a pivotal role in this phenomenon.

Anti-CD44 and anti-TLR4 blocking Abs reduce IRAK-M mRNA expression in human monocytes stimulated with HA or exposed to A431 cells

Given the influence of HA on IRAK-M expression, we hypothesized that the mechanism of tumor-induced IRAK-M expression in human monocytes might involve the major HA receptor, CD44. To study this possibility, cultures of human monocytes were preincubated with a blocking anti-CD44 Ab for 1 h and then exposed to either HA for 3 h or the A431 cancer cell line for 6 h. In addition, we also analyzed the role of TLR4 in IRAK-M signaling by performing a similar experiment but using an anti-TLR4 blocking Ab. A clear dose-dependent inhibition of the HA stimulatory effect was observed, and both Abs impaired HA-induced expression of IRAK-M (Fig. 8). Moreover, human monocytes pre-exposed to anti-CD44 and/or anti-TLR4 blocking Abs for 1 h and then cocultured with A431 for 6 h expressed significantly reduced levels of IRAK-M mRNA compared with untreated cells (Fig. 9*A*). Both anti-CD44 and anti-TLR4 abolished the expression of IRAK-M induced by the tumor cell line in a dose-dependent manner. Interestingly, a clear synergism was observed when both Abs were simultaneously used. In striking contrast, an anti-TLR2 blocking Ab did not affect IRAK-M induction neither by purified HA nor by the A431 cell line. None of these treatments significantly induced apoptosis (<2% cells), as assessed by Annexin V/PI staining (Fig. 9*B*).

Monocytes from cancer patients express IRAK-M mRNA

To determine the relevance of our findings in vivo, we analyzed the expression of IRAK-M in CD14$^+$ monocytes from patients

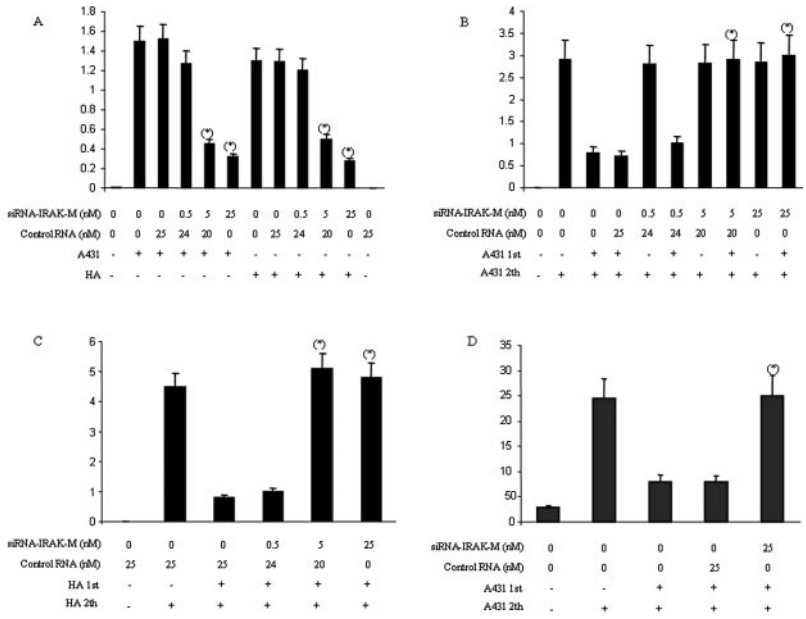

FIGURE 7. Specific IRAK-M down-regulation reinstated TNF-α mRNA and protein expression in human monocytes re-exposed to cancer cell line A431 or HA. Cultures of human monocytes were transfected with an IRAK-M–specific siRNA or control RNAs at indicated concentrations and incubated for 20 h. *A*, Transfected monocytes were stimulated with 100 μg/ml HA for 6 h or cocultured with A431 cells (ratio 1:0.3) for 6 h. After this period, cultures were washed three times with PBS and the cells were harvested. When cocultured, CD14$^+$ were recovered. The total RNA were isolated and cDNA synthesized. IRAK-M/18S gene expression was analyzed by real-time Q-PCR. *, $p < 0.05$ with respect to nontransfected cultures ($n = 3$). *B*, Transfected and control cultures of human monocytes were cocultured or not with A431 (ratio 1:0.3) for 6 h (A431 1st), washed and the recovered CD14$^+$ cells were re-exposed to A431 (ratio 1:0.3) for 2 h (A431 2th). CD14$^+$ cells were recovered again and total RNA isolated. TNF-α/18S gene expression was analyzed by real-time Q-PCR. *, $p < 0.05$ with respect to nontransfected cultures ($n = 3$). *C*, Transfected cells were stimulated or not with 100 μg/ml HA for 6 h (HA 1st) after this, cultures were washed three times with PBS and restimulated with 100 μg/ml HA for 2 h (HA 2th). Total RNA was isolated and cDNA synthesized. TNF-α/18S gene expression was analyzed by real-time Q-PCR. *, $p < 0.05$ with respect to cultures transfected with control RNA only ($n = 3$). *D*, Levels of TNF-α in the supernatants of 18-h cocultures were determined by ELISA ($n = 3$).

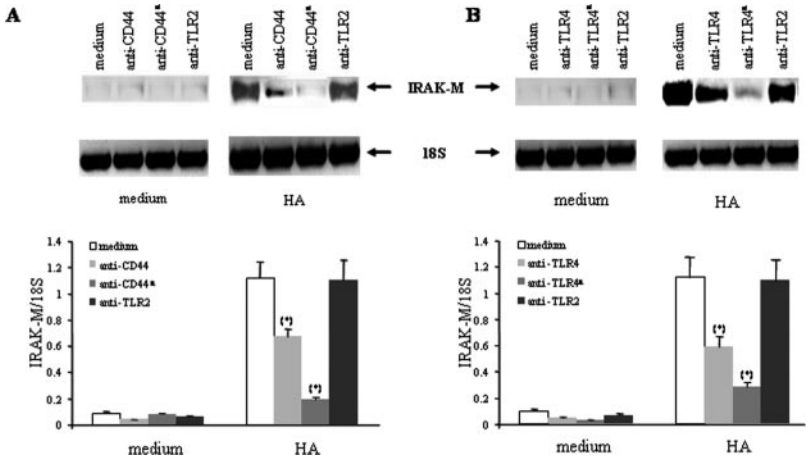

FIGURE 8. Anti-CD44 and anti-TLR4 blocking Abs abolish HA-induced IRAK-M expression in human monocytes. *A*, Human monocytes were pretreated with either 0.1 μg/ml (light gray bars) or 10 μg/ml (dark gray bars) anti-CD44 Ab, 10 μg/ml anti-TLR2 Ab (filled bars) or culture medium (open bars) for 1 h. The cultures were washed five times with PBS and stimulated with 100 μg/ml HA for 3 h. Total RNA was then isolated and the ratio of IRAK-M/18S mRNA was assessed by real-time Q-PCR. Typical results are shown ($n = 4$) and (*) $p < 0.05$ with respect to HA-treated cultures. The Q-PCR end products were analyzed by agarose gel electrophoresis (*upper panel*). *B*, Human monocytes were pretreated with either 0.5 μg/ml (light gray bars) or 5 μg/ml (dark gray bars) anti-TLR4 Ab, 10 μg/ml anti-TLR2 Ab (filled bars) or culture medium (open bars) for 1 h. The cultures were washed five times with PBS and stimulated with 100 μg/ml HA or medium for 3 h. The ratio of IRAK-M/18S mRNA was determined by real-time Q-PCR. Typical results are shown ($n = 3$); *, $p < 0.05$ with respect HA treated cultures. The Q-PCR end products were resolved by agarose gel electrophoresis and stained with ethidium bromide (*upper panel*).

with different tumors (Fig. 10). $CD14^+$ monocytes of all six patients with chronic myeloid leukemia expressed large amounts of IRAK-M mRNA. In contrast, very low levels, if any, of TNF-α mRNA were detected. Similarly, two patients with metastasis were also analyzed and the IRAK-M levels were elevated in both, while no TNF-α mRNA was detected in these patients. Finally, two other patients with localized, nonmetastatic colon tumors were included in our study, and in contrast with the patients with metastasis or leukemia, no IRAK-M mRNA was detected.

Discussion

Despite extensive investigations on the monocyte response to cancer cells, the data available are not sufficient to complete the picture of the molecular mechanisms underlying this phenomenon. When monocytes/macrophages contact a tumor, they activate an inflammatory response within a few hours (12, 40, 41). Nevertheless, monocytes become deactivated after their first exposure to cancer cells (12, 41). This kind of tolerance is characterized by a down-regulation in the expression of several cytokines, and might be termed "cancer-induced tolerance." Indeed, the transcription of TNF-α, IL-12, and other proinflammatory molecules is negatively regulated following the initial monocyte contact with tumor cells (12).

Our current observations confirm these previous reports regarding down-regulation of inflammatory responses when monocytes are re-exposed to a particular tumor cell line. In our hands, the

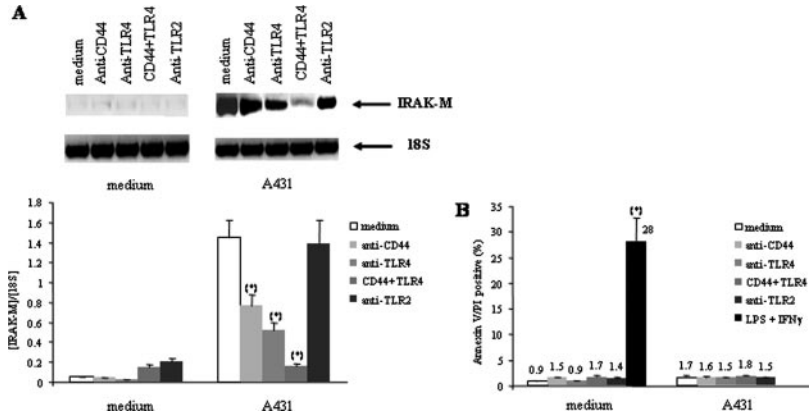

FIGURE 9. Effect of anti-CD44 and anti-TLR4 blocking Abs on A431-induced IRAK-M expression in human monocytes. *A*, Human monocytes were pretreated with either 0.1 μg/ml anti-CD44 (light gray bars), 0.5 μg/ml anti-TLR4 (dark gray bars), or with both Abs at the same final concentrations as above (dashed bars), 10 μg/ml anti-TLR2 (black bars) or in culture medium (open bars) for 1 h. Cells were washed five times with PBS and cocultured with A431 (ratio 1:0.3) or medium for 6 h. The $CD14^+$ monocytes were then recovered and the ratio of IRAK-M/18S mRNA was determined by real-time Q-PCR. A representative experiment is shown ($n = 4$) and (*) $p < 0.05$ with respect to the activation with A431. The Q-PCR end products were analyzed by agarose gel electrophoresis (*upper panel*). *B*, Human monocytes were pretreated with anti-CD44, anti-TLR4, both Abs, anti-TLR2, or cultured in medium for 1 h. Cells were then incubated with A431 (ratio 1:0.3) for 20 h. Then, $CD14^+$ monocytes were recovered and resuspended in PBS. The cells were double-stained with Annexin V/PI and analyzed by flow cytometry. The proportion of cells stained for Annexin V/PI is given ($n = 3$). *, $p < 0.05$ with respect to the control (no treatment). As a positive control we used monocytes incubated with 10 μg/ml LPS, 100 U/ml IFN-γ (LPS/IFN-γ) for 20 h.

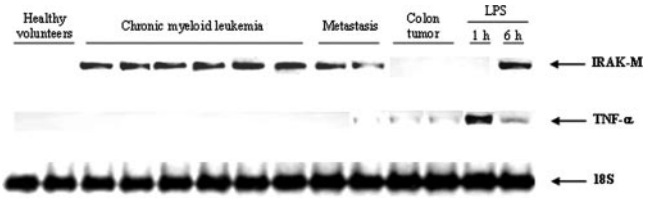

FIGURE 10. IRAK-M and TNF-α transcription analysis in human monocytes from cancer patients. CD14$^+$ monocytes from patients with the pathologies indicated and from healthy volunteers were isolated using the LS system. The total RNA was isolated and cDNA synthesized. The ratios of IRAK-M/18S and TNF-α/18S were determined by real-time Q-PCR. Cultures of human monocytes treated with 10 ng/ml LPS for 1 and 6 h were used as controls of IRAK-M and TNF-α expression, respectively. The Q-PCR end products were resolved by agarose gel electrophoresis and stained with ethidium bromide ($n = 2$).

inflammatory responses of human monocytes exposed to six different human cancer cell lines were down-regulated in their second encounter with tumor cells. In addition, we report for the first time that this event correlates with a strong up-regulation of IRAK-M mRNA levels, which peaked around 6 h after monocytes encountered cancer cells. Further, A431–pre-exposed monocytes rapidly ($\approx$2 h) overexpressed IRAK-M when they were re-exposed to the same cell line. These findings indicate the existence of a molecular mechanism that permits IRAK-M to be rapidly transcribed in the presence of a tumor within the framework of cancer tolerance. This effect appears to be highly specific for tumor cells, as no change in IRAK-M expression was detected when monocytes were exposed to human fibroblasts or lymphocytes. Moreover, pre-exposure to a nontumor cell did not accelerate IRAK-M mRNA expression when the monocytes were subsequently exposed to a cancer cell line.

We and others have previously demonstrated a role of the IRAK-M protein in deactivation of the inflammatory responses (32–36). In particular, we have shown that IRAK-M is rapidly up-regulated in the presence of LPS in human monocytes within the framework of endotoxin tolerance (33). In this study, in the context of cancer-induced monocyte tolerance, IRAK-M was also rapidly expressed when monocytes re-encountered a particular tumor. In addition, tolerance was abolished when IRAK-M expression was blocked by a specific siRNA. These data suggest the existence of a general mechanism that governs the unresponsiveness state in human monocytes, and which appears to involve IRAK-M expression as a crucial step.

Our findings also indicate that both a membrane-bound molecule from tumor cells and a soluble factor released by these cells induce transcription of the IRAK-M gene in human monocytes, because IRAK-M was up-regulated when human monocytes were cultured both in the presence of fixed tumor cells and in medium supplemented with the supernatant from cancer cell cultures. Interestingly, IRAK-M transcription was induced more rapidly in these experiments than when monocytes were cocultured with tumor cells. This observation points to the presence of a factor in the latter cocultures, which delays the signaling cascade that eventually culminates in IRAK-M expression in human monocytes.

It is well known that cancer cells overexpress and secrete large amounts of HA (7, 8, 42). The expression of this glycosaminoglycan correlates with an unfavorable prognosis in several tumors (8, 39, 42). Furthermore, HA interacts with different molecules expressed on the outer membrane of several cells and promotes intracellular signaling (12, 39, 42, 43). Our data show that incubation with LPS-free, purified HA induces the transcription of IRAK-M in human monocytes, a 3-h exposure to HA being sufficient to produce a noticeable effect. In addition, HA was able to induce TNF-α mRNA expression in a time-dependent manner. Moreover, the observed effects on both IRAK-M and TNF-α expression were abolished when HA degrading enzymes were used (data not shown). The increase in the expression of IRAK-M transcripts was accompanied by significant changes at the protein level in all conditions we tested. The protein was detected after 6–8 h and at least until 24 h of coculture with tumor cells.

One of the most important pathways in the inflammatory responses of monocytes is triggered when TLR family members recognize specific pathogen-associated molecular patterns. This is followed by the recruitment of intracellular adaptor proteins such as MyD88 and TIR domain-containing adaptor protein/Mal and the formation of a complex between IRAK-1, IRAK-4, and TNFR-associated factor 6 around these adaptors. The nonfunctional kinase IRAK-M might block this signaling pathway by binding to the latter complexes (35), thus inhibiting phosphorylation and *trans* activation events necessary for the activation of NF-κB and the resulting changes in gene expression. Although this hypothesis remains to be confirmed, we have clearly demonstrated that IRAK-M protein accumulates in human monocytes during cancer tolerance.

The mechanism by which tumor cells induce IRAK-M in human monocytes is unknown. In a previous study, we demonstrated that PI3K is involved in the control of IRAK-M expression in the context of endotoxin tolerance (33). In this study, we show that HA also induces this protein in human monocytes. Activation of the TLR4 pathway by LPS traces was ruled out, as all HA solutions were proved to be free of endotoxin contamination. Moreover, HA-preincubated monocytes showed a significant deactivation when rechallenged with HA. However, this effect was abolished in IRAK-M siRNA-transfected cultures (Fig. 7*C*). In contrast, preincubation of human monocytes with blocking Abs against CD44 and TLR4 significantly reduced the effect of HA on IRAK-M induction. Both, CD44 and TLR4 can bind to HA and they are expressed in several cell types including monocytes (44–49). CD44, which constitutes the major receptor for HA, interacts through its cytoplasmic domain with members of the erzin family, forming a complex that is involved in diverse physiological and pathological processes, in particular in tumor cells (43). We have provided evidence that not only TLRs but also CD44 is able to induce IRAK-M expression (36). In addition, when human monocytes were preincubated with either anti-CD44 or anti-TLR4 Abs and then cocultured with A431, the transcription of IRAK-M was clearly reduced and a synergism between both Abs was observed. These data suggests that both molecules participate in the interaction of HA with human monocytes and in the subsequent induction of IRAK-M expression. The effects observed on the expression of IRAK-M in human monocytes cocultured with tumor cells seem to be due to both membrane-bound HA and fragments released by cancer cells.

The study of IRAK-M transcripts in monocytes (CD14$^+$) from patients with circulating tumor cells substantiate our data. Monocytes isolated from patients who suffer chronic myeloid leukemia express large amounts of IRAK-M mRNA. These cells are in continuous contact with tumor cells and, as we predicted from the results of our in vitro model, express significant levels of IRAK-M. In contrast, TNF-α mRNA was not detected in these cells. In addition, we analyzed patients with metastasis and patients with localized solid colon tumors. Monocytes from the first group of patients expressed IRAK-M and very little TNF-α mRNA. In contrast, neither healthy individuals nor patients with solid colon cancers but without detectable metastasis expressed IRAK-M transcripts in their blood monocytes.

In conclusion, the data present in this study indicate that IRAK-M is involved in the development of tolerance to cancer cells in human monocytes. IRAK-M expression was blocked using

siRNA techniques, and as expected, precoculture with A431 did not induce tolerance in human monocytes. In addition, we have provided clear evidence of IRAK-M up-regulation in monocytes from chronic myeloid leukemia patients and patients that are undergoing metastasis. The mechanism by which IRAK-M is activated in this situation is unknown. Nevertheless, our findings suggest that HA may play an important role in the induction of the cancer tolerance state in human monocytes, because this molecule stimulates IRAK-M expression in human monocytes through the activation of CD44 and TLR4, but not TLR2.

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Disclosures

The authors have no financial conflict of interest.

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