

## Toll-like Receptor-4 Is Up-Regulated in Hematopoietic Progenitor Cells and Contributes to Increased Apoptosis in Myelodysplastic Syndromes

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**Abstract** **Purpose:** To investigate the function and expression of Toll-like receptors (TLR) in bone marrow cells of myelodysplastic syndrome (MDS) patients and to examine their involvement in the apoptotic phenomenon characterizing MDS hematopoiesis. **Experimental Design:** TLR mRNA and protein expression was investigated in bone marrow cell populations of MDS patients and controls. TLR-4 ability to recognize lipopolysaccharide and up-regulate self mRNA and protein expression was examined. Tumor necrosis factor involvement in the constitutive and lipopolysaccharide (LPS)-induced TLR expression was also evaluated. Possible correlation between TLR-4 overexpression and apoptosis was investigated by simultaneous staining with Annexin V and TLR-4. **Results:** TLR-2 and TLR-4 are expressed in almost all bone marrow cell lineages including megakaryocytes, erythroid cells, myeloid precursors, monocytes, and B lymphocytes and are up-regulated in MDS patients compared with controls. In hematopoietic CD34<sup>+</sup> cells, TLR-4 is also expressed and significantly up-regulated at both the mRNA and protein levels. Treatment with an anti-tumor necrosis factor antibody reduces both constitutive and LPS-induced TLR-4 levels. Increased TLR-4 expression correlates with increased apoptosis as TLR-4 is almost exclusively found in apoptotic bone marrow mononuclear and CD34<sup>+</sup> cells. The addition of the TLR-4 ligand LPS further enhances the apoptosis of these cells. **Conclusions:** TLR-4 and other TLRs are significantly up-regulated in MDS patients whereas TLR-4 is involved in promoting apoptosis, possibly contributing to MDS cytopenia.

The Toll-like receptors (TLR) are members of a conserved family of type I transmembrane receptors characterized by an intracellular signaling domain homologue to the interleukin (IL)-1 receptor (1, 2). On ligation, TLR signaling triggers the expression of proinflammatory cytokines, chemokines, and costimulatory and adhesion molecules, leading to the priming of the adaptive immune system and initiation of inflammatory responses (3–5). Expression of TLRs is induced by microbial invasion and microbial components (6, 7). To date, the mammalian TLR family is known to consist of 11 members (TLR1-TLR11), each having specificity to various bacterial, fungal, and viral elements (6, 8). Furthermore, self molecules

including heat shock proteins, DNA/RNA from dying cells, fibronectin fragments, and fibrinogen released in response to stress, tissue damage, and cell death have all been shown to act as ligands for a number of TLRs (8). Cytokines such as IFN $\gamma$ , tumor necrosis factor (TNF)- $\alpha$ , interleukins (IL-1 $\beta$ , IL-2, and IL-5), and growth factors have thus far been implicated in either increased or decreased TLR expression in various cell types. More specifically, IFN $\gamma$  and TNF have been shown to induce the expression of TLR-2 and TLR-4 in renal epithelium (9).

TLR triggering has been linked in the past to excessive programmed cell death through the production of various cytokines. Recent studies have, however, suggested a direct relationship between TLR-4 and apoptosis, showing that stimulation of overexpressed TLR-4 promoted death in epithelial cells. A dominant-negative mutant of a Fas-associated death domain protein could suppress TLR-4-mediated cell death, indicating that TLR-4 may directly induce apoptosis through a Fas-associated death domain protein-dependent pathway (10–12).

Myelodysplastic syndromes (MDS) constitute a heterogeneous family of clonal disorders of the hematopoietic progenitor cell, predominantly displaying ineffective hematopoiesis and peripheral cytopenias (13). The cause of cytopenias, in at least one subset of MDS patients, has been attributed to an excessive cytokine-induced intramedullary apoptotic death (14, 15). The levels of several cytokines or ligands, known to have proapoptotic and inflammatory properties such as IL-1 $\beta$ , TNF, and Fas

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**Table 1.** Demographic, clinical, and hematologic characteristics of patients

Patient	Age/sex	WHO	Hemoglobin (g/dL)	Neutrophil ( $\times 10^3/\text{mm}^3$ )	Platelet ( $\times 10^3/\text{mm}^3$ )	IPSS	Karyotype
1	76/F	RA	9.5	3.5	240	Int-1	46XX
2	88/M	RAEB-I	8.4	0.5	82	Int-2	Inv 9
3	72/M	RAEB-II	8.5	0.6	40	High	2q-, 7q-, 18-
4*	71/M	RAEB-II	5.6	0.8	16	High	46XY
5	79/M	RAEB-I	7.1	0.3	50	Int-2	46XY
6*	88/M	RAEB-I	7.4	0.4	60	Int-2	46XY
7*	80/F	RA	10.0	2.7	168	Int-1	46XX
8*	78/F	RAEB-II	8.0	0.1	10	High	7-
9*	78/M	RA	8.0	2.5	80	Int-1	46XY
10*	65/F	RA	10.5	2.7	230	Int-1	46XX
11*	75/M	RA	10.9	3.4	270	Low	46XY
12*	72/F	RAEB-I	8.9	2.0	90	Int-2	20q-, 8+
13	70/F	RCMD	7.4	2.0	68	Int-1	46XX
14*	56/F	RA	9.0	7.0	352	Low	46XX
15*	71/M	RAEB-II	8.7	1.8	56	High	Inv 9
16*	68/F	RA	8.0	3.6	230	Int-1	46XX
17*	80/M	RCMD	8.0	0.7	82	Int-1	46XY
18*	73/F	RAEB-I	9.7	2.5	100	Int-2	8+
19*	65/M	RCMD	10.5	2.9	120	Low	46XY
20*	65/F	RAEB-I	9.0	3.5	353	Int-2	46XX
21*	72/F	RAEB-I	6.2	1.2	67	High	5q-, 8+

\*Patients studied by flow cytometry.

ligand, are elevated in myelodysplastic bone marrow (16–20). This elevated expression has been implicated in increased apoptosis and inhibition of hematopoiesis. It has been hypothesized that TNF primes bone marrow CD34<sup>+</sup> cells for Fas-induced apoptosis, suggesting a Fas-Fas ligand interaction as a possible pathogenetic mechanism contributing to immune destruction of CD34<sup>+</sup> cells in human myelodysplasia (17).

Taking into consideration mounting evidence supporting the involvement of TLRs in the apoptotic process, we reasoned that measurement of TLR expression levels and their functional ability in MDS-derived cells could provide a further insight into the pathophysiologic events leading to increased apoptosis.

## Materials and Methods

**Patient characteristics.** Twenty-one newly diagnosed, primary MDS patients were enrolled in the study. The patients were classified according to the WHO criteria and further categorized consistent with the International Prognostic Scoring System (IPSS; Table 1; ref. 13). Twenty uncomplicated iron deficiency anemia patients (9 females and 11 males) were used as controls. None of the patients or controls had experienced microbial infection at least 6 months before the bone marrow aspiration. Informed consent was obtained from all participants in accordance with the regional Hospital Ethical Committee.

**Specific reagents.** Phycoerythrin-conjugated human anti-TLR-4 (clone HTA125) and anti-TLR-2 (clone TL2.1) were sourced from

eBioscience (San Diego, CA) and immunoglobulin G (IgG; isotype control) monoclonal antibody was acquired from Santa Cruz Biotechnology (Santa Cruz, CA). FITC-conjugated human antibodies against CD41, CD71, CD33, CD14, CD20, CD3, and Annexin V, as well as phycoerythrin-conjugated antibody against human intercellular adhesion molecule-1 (ICAM-1), were obtained from Becton Dickinson (San Jose, CA). LPS (*E. coli*, serotype O55:B5) was purchased from Sigma (St. Louis, MO). To ensure the specific function of the chimeric anti-TNF antibody (infiximab), we used total human IgG and a mouse IgG antibody as controls. Titration experiments were done to determine the optimal concentration and induction time of all the reagents used in the experimental protocols.

**Bone marrow mononuclear cell and CD34<sup>+</sup> cell isolation and THP-1 cell cultures.** Bone marrow mononuclear cells (BMMC) were isolated from fresh bone marrow samples by density gradient centrifugation using Ficoll Hypaque (specific gravity, 1.077 g/dL; Amersham Bioscience, Uppsala, Sweden). CD34<sup>+</sup> cells were obtained by immunomagnetic separation on Mini Macs columns (Miltenyi Biotec, Inc., Gladbach, Germany), with purity >95% as assessed by flow cytometry. The neoplastic peripheral blood monocytic cell line THP-1, used as positive control, was cultured in RPMI 1640 (Life Technologies, Inc., Grand Island, NY) supplemented with 10% fetal bovine serum,  $5 \times 10^{-5}$  mol/L 2-mercaptoethanol, 2 mmol/L L-glutamine, 100 units/mL penicillin, and 100  $\mu\text{g}/\text{mL}$  streptomycin.

**TLR mRNA analysis.** TLR-1 to TLR-4 mRNA expression was examined in patients, controls, and THP-1 cells. Total RNA was extracted from  $0.5 \times 10^6$  to  $2 \times 10^6$  BMMCs, CD34<sup>+</sup> cells, and THP-1 cells using Trizol RNA isolation kit (Invitrogen Life Technologies,

**Table 2.** Characteristics of primer sets and PCR used to detect 18S rRNA and TLRs

Target gene	Accession no.	Amplicon position	Amplicon size	Amplification efficiency
18S rRNA	NG 002801	1,151-1,453	302	1.98
TLR-1	NM_003263	1,829-2,117	289	1.94
TLR-2	NM_003264	1,759-2,102	344	1.96
TLR-3	NM_0032635	2,407-2,771	362	1.87
TLR-4	NM_003266	3,035-3,370	355	1.91

Paisley, United Kingdom), and 1  $\mu$ g was reverse transcribed into cDNA using the Promega reverse transcription-PCR kit (Promega, Madison, WI) and oligo-dT primers according to the manufacturer's instructions. Real-time PCR was used to confirm the integrity and to carry out the normalization of all cDNA samples through the amplification of the 18S rRNA, which served as the housekeeping gene.

**Quantitative real-time PCR.** TLR mRNA levels were quantified by real-time PCR that was done on a LightCycler system (Roche, Molecular Biochemicals, Mannheim, Germany). We designed oligonucleotide primers for each mRNA, consistent with the published sequences (Table 2). Absolute quantification with the use of SYBR Green 1 dye and standard curves was applied as previously described (21).

**Flow cytometry.** TLR protein expression in BMMCs and CD34<sup>+</sup> cells, as well as in THP-1 cells, was examined by flow cytometry analysis, either constitutively or after the appropriate stimulation. Flow cytometry analysis incorporated 16 of the MDS patients (6 RA, 2 RCMD, 5 RAEB-I, and 3 RAEB-II) but, for homogeneity reasons, only 8 of the low/Int-1 patients (6 RA and 2 RCMD) entered the apoptosis protocol (Table 1). Dual staining was applied using antibodies against cell lineage-specific markers [megakaryocytes (CD41), erythroid cells (CD71), myeloid precursors (CD33), monocytic lineage (CD14), B lymphocytes (CD20), and T lymphocytes (CD3)] and TLR-4 or TLR-2 to evaluate the presence and distribution of the two receptors in each bone marrow hematopoietic differentiation compartment. Apoptosis was measured in BMMCs and CD34<sup>+</sup> cells by dual staining with TLR-4-phycoerythrin and Annexin V-FITC antibodies. Cells were analyzed using a FACSCalibur flow cytometer and CELLQuest software (Becton Dickinson) with appropriate forward and side scatter adjustments. The results of single staining were evaluated both as mean fluorescence intensity and positive percentage of the total population with comparable results. Dual staining was evaluated as positive percentage of the total population.

**Functional assessment of TLRs.** TLR-4 ability to recognize LPS (TLR-4 ligand) and induce up-regulation of self mRNA and protein expression, as well as ICAM-1 protein expression, was examined. BMMCs and CD34<sup>+</sup> cells from MDS patients and controls, as well as THP-1 cells, were exposed to serum-free medium alone or serum-free medium containing 1  $\mu$ g/mL LPS. The implication of LPS in the apoptosis of MDS cells was also assessed by Annexin V assay, both constitutively and after LPS and anti-TNF modulation. To evaluate the TNF implication in both TLR-4 and ICAM-1 expression, as well as in the constitutive or LPS-induced apoptosis, MDS BMMCs and THP-1 cells were exposed to serum-free medium alone or serum-free medium containing recombinant human TNF (200 IU/mL), anti-TNF antibody (infliximab; 10  $\mu$ g/mL), and concurrent LPS and anti-TNF antibody.

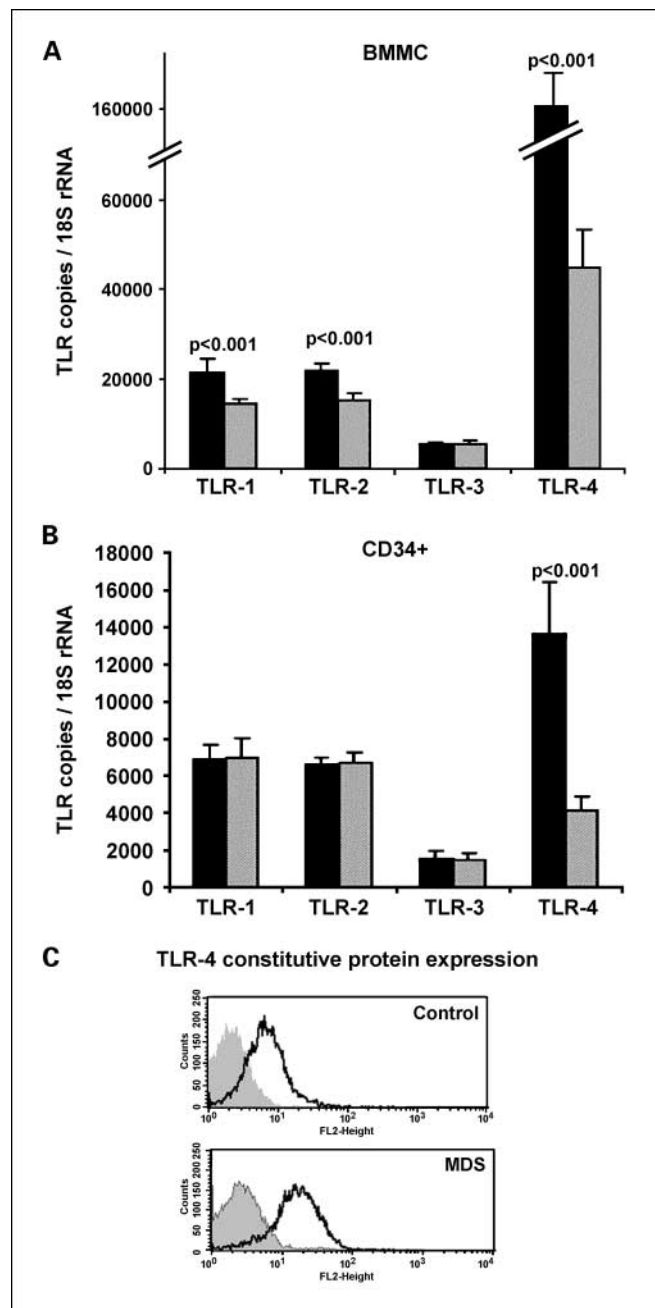
**Statistical analysis.** Continuous variables were compared using the Mann-Whitney *U* test. Ordered categorical variables were correlated using Spearman's  $\rho$ . Significance was set to 0.05. Stata V8 was used for analysis.

## Results

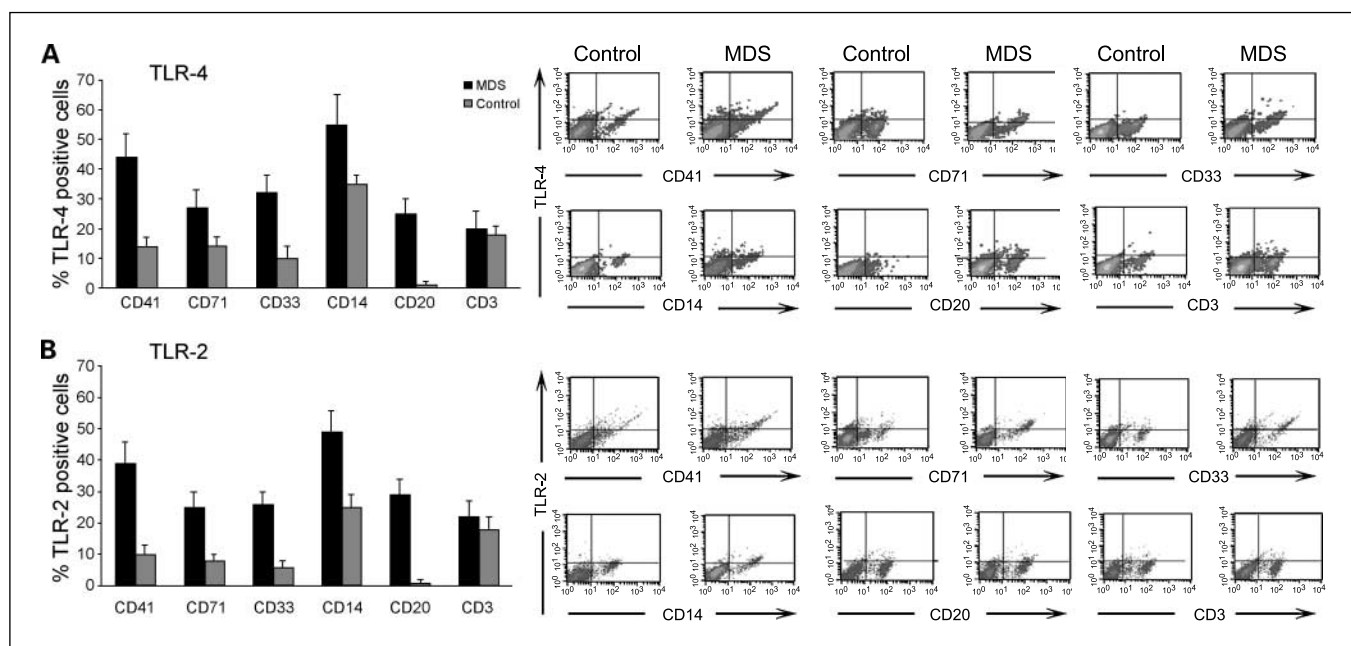
### TLR-2 and TLR-4 expression in BMMCs and CD34<sup>+</sup> MDS cells.

We first examined the expression of TLR-1, TLR-2, TLR-3, and TLR-4 mRNA by reverse transcription-PCR (data not shown) and quantitative real-time PCR. We observed that although TLR-1, TLR-2, TLR-3, and TLR-4 mRNA were found in both MDS and control patients, MDS BMMCs had significantly higher levels of TLR-1 ( $P < 0.001$ ), TLR-2 ( $P < 0.001$ ), and TLR-4 mRNA ( $P < 0.001$ ; Fig. 1A). TLR-3 mRNA was found to be low in BMMCs of both patients and controls. Interestingly, when the CD34<sup>+</sup> subset was examined, MDS patients displayed significantly higher levels of TLR-4 mRNA compared with controls ( $P < 0.001$ ) whereas no statistically significant difference was noted in levels of TLR-1 and TLR-2 (Fig. 1B).

This indicates that overexpression of TLR-1 and TLR-2 in BMMCs is the result of their overexpression in the CD34<sup>-</sup> compartment. Flow cytometric analysis revealed that MDS BMMCs expressed  $19 \pm 2.7\%$  higher levels of TLR-4 protein and  $14 \pm 2.2\%$  TLR-2 protein compared with controls ( $P < 0.01$ ), a finding consistent with mRNA expression studies



**Fig. 1.** TLR-1, TLR-2, TLR-3, and TLR-4 expression in BMMCs and CD34<sup>+</sup> cells from MDS patients and controls. **A**, TLR-1, TLR-2, TLR-3, and TLR-4 mRNA expression in BMMCs. **B**, TLR-1, TLR-2, TLR-3, and TLR-4 mRNA expression in CD34<sup>+</sup> cells. **A** and **B**, columns, mean TLR copies normalized against 18S rRNA (black columns, MDS patients; gray columns, uncomplicated iron deficiency controls); bars, SD. MDS BMMCs express higher levels of TLR-1, TLR-2, and TLR-4 mRNA in comparison with controls. In CD34<sup>+</sup> cells, only TLR-4 is significantly up-regulated in MDS patients. **C**, TLR-4 protein expression in BMMCs in both patients and controls as examined by flow cytometry. Data show higher TLR-4 constitutive protein expression from a representative MDS patient in comparison with a control. Gray, isotype control staining; black, TLR-4-positive staining.



**Fig. 2.** TLR-4 and TLR-2 presence in lineage specific bone marrow cell populations. *A*, TLR-4 protein expression in specific bone marrow cell population. *B*, TLR-2 protein expression in specific bone marrow cell population. *A* and *B*, black columns, MDS patients; gray columns, uncomplicated iron deficiency controls. Megakaryocytic (CD41), erythroid (CD71), myeloid (CD33), and monocytic (CD14) cell subsets of MDS patients display substantial increase of TLR-2 and TLR-4 expression compared with the uncomplicated iron deficiency controls. Control CD20<sup>+</sup> B-lymphocytes have very low TLR expression in contrast to the MDS B cells. A marginal increase is observed in MDS CD3<sup>+</sup> T lymphocytes. The fluorescence-activated cell sorting plots represent TLR-4 and TLR-2 protein expression, respectively, in specific bone marrow cell subsets from one patient and one control (*A* and *B*).

(Fig. 1C). Moreover, in MDS CD34<sup>+</sup> cell population, TLR-4 protein levels were increased by 92% compared with controls ( $P < 0.01$ ).

TLR-4 and TLR-2 protein expression was also evaluated in CD34<sup>-</sup> bone marrow cells both in patients and controls. Compared with controls, TLR-2 and TLR-4 expression in MDS patients was significantly up-regulated ( $P < 0.01$ ) in almost all bone marrow cell populations examined, including megakaryocytes, erythroid cells, myeloid precursors, monocytes, and B lymphocytes (Fig. 2A and B). The highest increase in TLR-4- and TLR-2-positive cells was observed in megakaryocytes (30% and 29%, respectively) whereas T lymphocytes were the only population showing no significant increase in the expression of these receptors in comparison with controls. The mean values of the percentage of TLR-2- and TLR-4-positive cells in each specific cell lineage in patients and controls are presented in Table 3.

To examine whether TLR expression correlates with disease severity, we evaluated its expression in two groups, one

consisted of low/Int-1 IPSS patients and the other of high/Int-2 patients. Neither group presented any statistically significant variation of TLR expression in either BMMCs or CD34<sup>+</sup> cells. The protein expression of TLR-4 and TLR-2 on erythroid cells was inversely correlated with hemoglobin levels ( $\rho = -0.53$ ,  $P = 0.034$  and  $\rho = -0.53$ ,  $P = 0.03$ , respectively). In addition, the protein expression of TLR-4 on monocytes was inversely correlated with hemoglobin ( $\rho = -0.60$ ,  $P = 0.01$ ), neutrophil ( $\rho = -0.76$ ,  $P = 0.001$ ), and platelet ( $\rho = -0.67$ ,  $P = 0.004$ ) levels.

**TLR-4 is functional and capable of up-regulating its own expression.** To assess whether TLR-4 is functional and capable of signaling, we used LPS to activate BMMCs. We found that LPS up-regulated ICAM-1, a surrogate marker for TLR-4-mediated activation, in a time- and dose-dependent manner (data not shown). LPS-mediated ICAM-1 induction was comparable between MDS patients and controls [13.7-fold ( $\pm 1.7$ ) versus 11.4-fold ( $\pm 1.1$ ), respectively]. In THP-1 cells, ICAM-1 expression increased by 82.2-fold ( $\pm 8.8$ ). We also

**Table 3.** Mean values of the percentage of positive cells from all samples examined in the study

	TLR-4			TLR-2		
	Control	MDS	% Increase	Control	MDS	% Increase
CD41	14 $\pm$ 3%	44 $\pm$ 8%	30	10 $\pm$ 3%	39 $\pm$ 7%	29
CD71	14 $\pm$ 3%	27 $\pm$ 6%	13	8 $\pm$ 25%	25 $\pm$ 5%	17
CD33	10 $\pm$ 4%	32 $\pm$ 6%	22	6 $\pm$ 2%	26 $\pm$ 4%	20
CD14	35 $\pm$ 3%	55 $\pm$ 10%	20	25 $\pm$ 4%	49 $\pm$ 7%	24
CD20	1 $\pm$ 1%	25 $\pm$ 5%	24	1 $\pm$ 1%	29 $\pm$ 5%	28
CD3	18 $\pm$ 3%	20 $\pm$ 6%	2	18 $\pm$ 4%	22 $\pm$ 5%	4

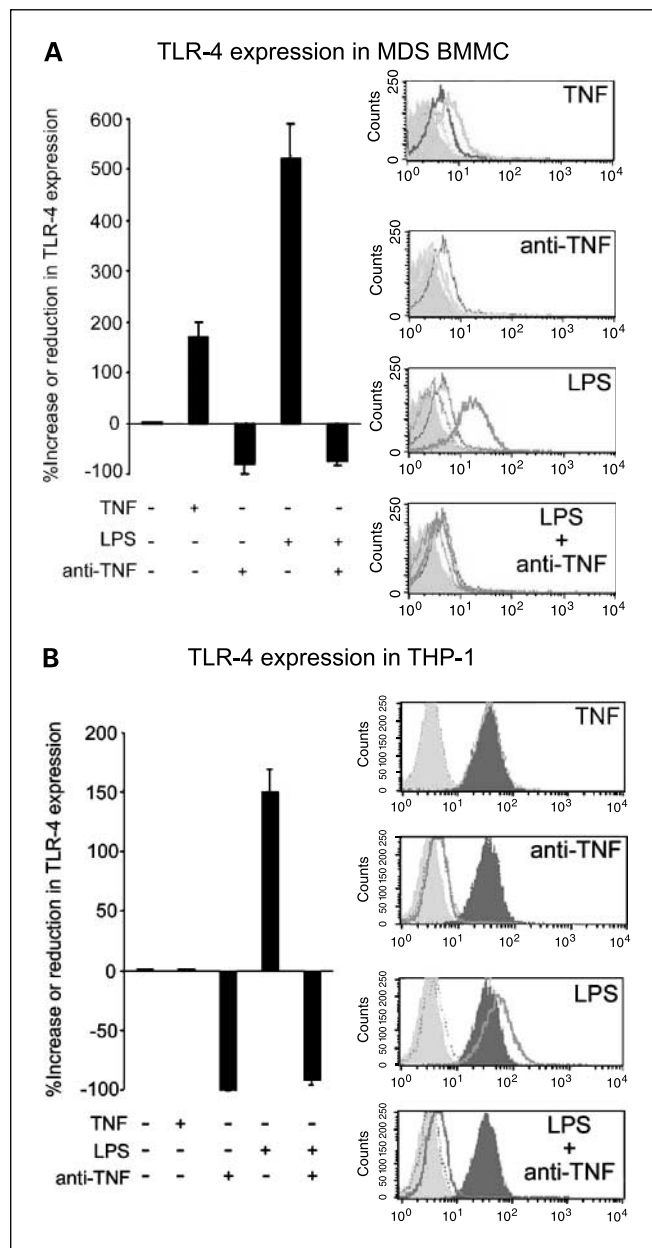


found that LPS powerfully up-regulated the expression of its receptor (TLR-4) with optimal TLR-4 mRNA expression being observed after 12 h of stimulation with 1.0  $\mu\text{g}/\text{mL}$  LPS. LPS increased TLR-4 mRNA levels by  $24 \pm 10\%$  in MDS BMMCs and by  $30 \pm 10\%$  in control BMMCs when compared with unstimulated cells. Similarly, LPS up-regulated TLR-4 mRNA levels by  $90 \pm 20\%$  in MDS CD34<sup>+</sup> cells and by  $70 \pm 20\%$  in control CD34<sup>+</sup> cells. With regard to protein levels, after 24-h incubation, LPS led to a  $520 \pm 70\%$  increase of TLR-4 protein expression in BMMCs derived from MDS patients and a  $340 \pm 50\%$  induction in BMMC from controls. THP-1 cells also revealed a  $150 \pm 20\%$  increase in TLR-4 protein levels following addition of LPS (Fig. 3).

**TLR-4 constitutive and LPS-induced levels are TNF mediated.** There is evidence that TLRs are regulated by cytokines such as TNF, IFN $\gamma$ , interleukins (IL-1 $\beta$ , IL-2, and IL-5), and growth factors. We therefore examined whether TNF regulates TLR-4 expression. We found that TNF up-regulated cell-surface TLR-4 by  $170 \pm 30\%$  in MDS BMMCs. Interestingly, constitutive TLR-4 levels were also found to be TNF dependent as the addition of infliximab, an anti-TNF monoclonal antibody, reduced TLR-4 levels by  $80 \pm 20\%$  in MDS BMMCs and 100% in THP-1. LPS stimulation with simultaneous blockade of TNF-mediated pathways showed a decrease in TLR-4 levels of  $75 \pm 10\%$  and  $91 \pm 5\%$  in MDS BMMCs and THP-1, respectively (Fig. 3).

**Apoptosis of BMMCs after LPS, TNF, and anti-TNF treatment.** Apoptosis was monitored by Annexin V binding assay constitutively or after addition of LPS, TNF, anti-TNF, and concurrent LPS and anti-TNF treatment. Constitutively the level of apoptosis after resting in serum-free medium for 24 h was found to be  $10 \pm 2.5\%$  and  $12 \pm 1.6\%$  of the total BMMC population in MDS and THP-1 cells, respectively, increasing to  $27 \pm 4.7\%$  and  $42 \pm 2.9\%$  ( $P < 0.001$ ) after LPS triggering. The addition of TNF also resulted in a statistically significant increase of apoptosis reaching  $19 \pm 3.1\%$  ( $P < 0.001$ ) and  $18 \pm 2.0\%$  ( $P < 0.001$ ) in BMMCs and THP-1, respectively. After anti-TNF treatment, however, the levels of apoptosis decreased in comparison with the constitutive levels in the two cell types ( $9 \pm 1.7\%$  and  $8 \pm 0.4\%$ , respectively). Finally, concurrent LPS induction and TNF blockade impelled the apoptotic levels to  $22.1 \pm 3.1\%$  ( $P < 0.001$ ) and  $35.0 \pm 2.7\%$  ( $P < 0.001$ ), revealing statistically significant reductions when compared with the LPS-driven apoptotic levels (Fig. 4).

**TLR-4 expression correlates with apoptotic cell death of CD34<sup>+</sup> cells and BMMCs.** To determine whether TLR-4 expression is correlated with the apoptosis of BMMCs and CD34<sup>+</sup> cells of MDS patients, we used the Annexin V binding assay. Due to the excessive heterogeneity of the high/Int-2 MDS subcategory, only low/Int-1 patients were evaluated. In eight of these patients,  $5.4 \pm 1\%$  of CD34<sup>+</sup> cells were found to be Annexin V<sup>+</sup> compared with  $0.5 \pm 0.4\%$  in controls. The Annexin V<sup>+</sup>/TLR-4<sup>+</sup> MDS CD34<sup>+</sup> cells were  $5.2 \pm 1\%$ , indicating that  $96 \pm 4\%$  of apoptotic cells express TLR-4 and  $71 \pm 6\%$  of the TLR-4<sup>+</sup> cells are apoptotic. In controls, only 8% of the apoptotic cells were TLR-4<sup>+</sup> (Fig. 5). Similarly, in BMMCs, we found that  $10 \pm 3\%$  of the resting cells were Annexin V<sup>+</sup> and  $8.3 \pm 2\%$  Annexin V<sup>+</sup>/TLR-4<sup>+</sup>, indicating that  $82 \pm 6\%$  of apoptotic cells express TLR-4 and  $73 \pm 8\%$  of the TLR-4<sup>+</sup> cells are apoptotic (Fig. 5). Addition of LPS significantly increased apoptotic Annexin V<sup>+</sup> cells to  $27 \pm 5\%$  ( $P < 0.001$ ) and Annexin V<sup>+</sup>/TLR-4<sup>+</sup> cells to  $24.6 \pm 4\%$  of the total BMMC

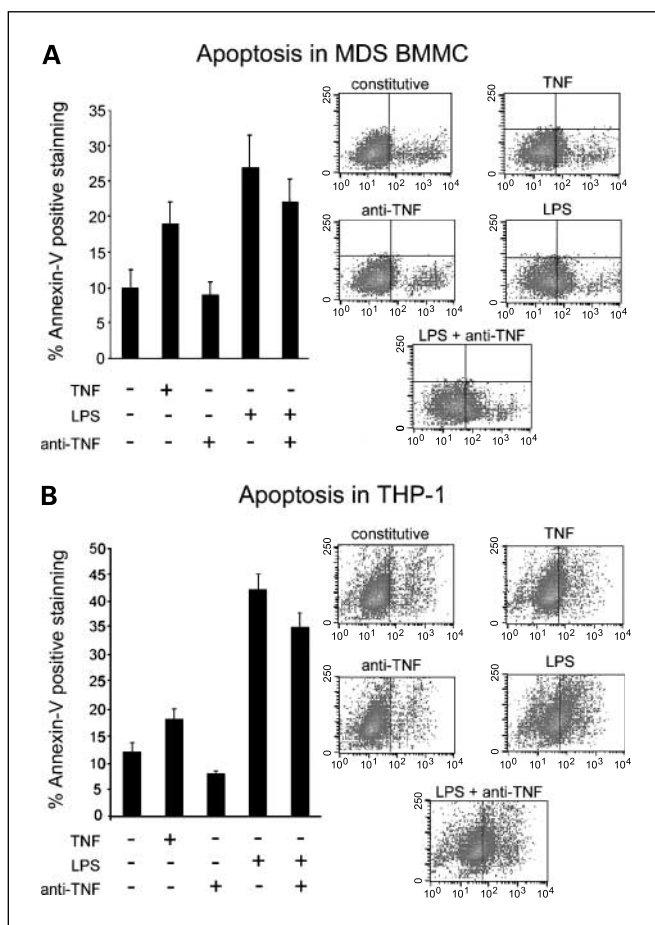


**Fig. 3.** TLR-4 expression is TNF regulated. **A**, TLR-4 protein expression in MDS BMMCs after LPS, TNF, and anti-TNF treatment. **B**, TLR-4 protein expression in THP-1 cells after LPS, TNF, and anti-TNF treatment. A severalfold increase of TLR-4 protein expression is found in MDS BMMCs following TNF or LPS treatment. TNF blockade reveals a total inhibition of TLR-4 expression in both BMMCs and THP-1 cells. The inhibitory effect of anti-TNF treatment is present even after the LPS challenge, indicating that TLR-4 expression is TNF dependent. The fluorescence-activated cell sorting plots (**A** and **B**) represent the alterations of TLR-4 expression in MDS BMMCs and THP-1 cells, respectively, following LPS, TNF, and anti-TNF treatment. +, addition of corresponding agent.

population. TNF treatment increased the number of both Annexin V<sup>+</sup> and TLR-4<sup>+</sup> BMMCs without affecting the percentage of Annexin V<sup>+</sup>/TLR-4<sup>+</sup> cells ( $8.5 \pm 2\%$ ).

## Discussion

To our knowledge, this report is the first to show that TLRs are abundantly expressed in the bone marrow of MDS patients. TLR-2 and TLR-4 are found in almost all cell lineages examined,



**Fig. 4.** Percentage of apoptosis as monitored by Annexin V binding assay. *A*, effect of LPS, TNF, and anti-TNF treatment on apoptosis in MDS BMMCs. *B*, the effect of LPS, TNF, and anti-TNF treatment on apoptosis in THP-1 cells. TNF and LPS are found to induce apoptosis in both MDS BMMCs and THP-1 cells. Anti-TNF treatment is associated with a marginal reduction of apoptosis in both cell types. Concurrent treatment with LPS and anti-TNF treatment also reduces the apoptotic levels compared with the LPS-driven levels in both cell types. The fluorescence-activated cell sorting plots (*A* and *B*) represent the alterations of apoptosis in MDS BMMCs and THP-1 cells, respectively, following LPS, TNF, and anti-TNF treatment. +, addition of corresponding agent.

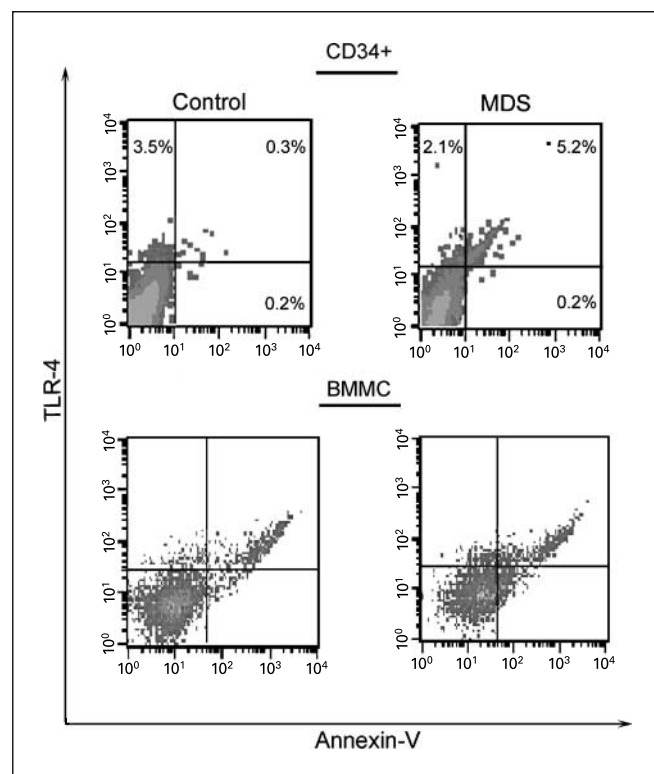
including megakaryocytes, erythroid cells, myeloid precursors, monocytic lineage, and B lymphocytes, and are significantly up-regulated in MDS patients when compared with controls. TLR-2 and TLR-4 were also detected in CD34<sup>+</sup> but, in contrast to other lineages, only TLR-4 was found to be significantly up-regulated in these cells. In addition, the up-regulated ICAM-1 expression by LPS indicates that TLR-4 is equally capable of signaling in MDS hematopoietic cells as in control cells. The higher expression of functional TLR-4 in patients reinforces the likelihood of its involvement in the pathogenesis of MDS because its aberrant increase is present not only in the differentiated cell types but also in the pathophysiologically important CD34<sup>+</sup> cell population.

Cytokines (TNF), growth factors, and endogenous ligands can all regulate TLR expression. We found that in MDS BMMCs, TLR-4 expression is TNF dependent as the addition of infliximab, an anti-TNF monoclonal antibody, strongly reduced both constitutive and LPS-induced TLR-4 levels, whereas the addition of TNF strongly up-regulated TLR-4 levels by severalfold. It is worth noting that TNF blockade seemed to

result in almost total TLR-4 inhibition. To our knowledge, this is the first time that anti-TNF antibodies have been shown to inhibit TLR-4 expression.

Aberrant TNF mRNA and protein expression has previously been found in MDS patients' bone marrow, and local cytokine production has been reported to correlate with the levels of intramedullary apoptosis (19, 20), expression of Fas antigen on blasts cells, and disease severity (22, 23). Although the pathophysiologic basis and the origin of TNF production in MDS remain unclear, its documented elevated expression in MDS bone marrow could explain the overexpression of TLR-4 on CD34<sup>+</sup> and on differentiated myeloid cells. Therefore, the increase in TLR-2 or TLR-4 expression can possibly be attributed to the elevated TNF and other cytokine production, although other factors, such as increased apoptosis, characterizing the syndrome cannot be excluded. It is known that MDS cells carry a number of genetic alterations that may affect the function of transcription factors (e.g., interferon regulatory factor-1 exon skipping; ref. 21) and, consequently, TLR and other gene expression.

Notably, TLR-4 expression correlates with the extent of apoptosis in bone marrow cells. MDS CD34<sup>+</sup> cells presented an ~5-fold and 2-fold increase in apoptosis and TLR expression, respectively. The TLR-4<sup>+</sup>/Annexin V<sup>+</sup> cell percentage in CD34<sup>+</sup> control cells increased from 8% to 96% in MDS cells. A strong correlation between apoptosis and increased TLR-4 expression



**Fig. 5.** TLR-4 expression strongly correlates with apoptotic cell death. Coexpression of TLR-4 and Annexin V in BMMCs and CD34<sup>+</sup> cells from MDS patients and controls. CD34<sup>+</sup> cells seem to be more apoptotic in MDS than in controls (Annexin V<sup>+</sup>: 5.4 ± 1% and 0.5 ± 0.4%, respectively). The Annexin V<sup>+</sup>/TLR-4<sup>+</sup> MDS CD34<sup>+</sup> cells are 5.2 ± 1%, indicating that 96 ± 4% of apoptotic cells express TLR-4 and 71 ± 6% of the TLR-4<sup>+</sup> cells are apoptotic. Similarly, MDS BMMCs are found to be more apoptotic than controls and a strong correlation between apoptosis and TLR-4 expression is also noted.

was also noted in MDS BMDCs. Furthermore, LPS triggering led to significantly increased apoptotic Annexin V<sup>+</sup> and Annexin V<sup>+</sup>/TLR-4<sup>+</sup> cells. Studies indicating that blockage of TLR signaling could reduce apoptosis in MDS cells may strengthen the conclusion that TLR-4 signaling contributes to increased apoptosis in MDS, providing a direct link between TLR-4 function and CD34<sup>+</sup> cell apoptosis.

Given the TNF elevated levels in MDS, the aptitude of TNF to induce TLR-4 expression, and the implication of TLR-4 in apoptosis, we suggest that increased TLR-4 expression may participate in MDS extensive apoptosis. However, the marked reduction in TLR-4 expression by anti-TNF was not associated with significant reduction in apoptosis, suggesting that a variety of other apoptotic mechanisms could also be responsible for the increased apoptosis in MDS.

What may trigger TLR signaling in MDS is as yet unknown. In addition to LPS, TLR-4 has been shown to recognize endogenous ligands such as heat shock proteins (HSP60 and HSP70), the extra domain A of fibronectins, oligosaccharides of hyaluronic acid, heparan sulfate, and fibrinogen (3, 5, 24, 25).

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