Reduced regulatory T cells (Treg) in bone marrow preferentially associate with the expansion of cytotoxic T lymphocytes in low risk MDS patients

The myelodysplastic syndromes (MDS) include clonal bone marrow (BM) disorders characterised by the emergence/dominance of dysplastic progenitors in the context of ineffective haematopoiesis, peripheral cytopenias and increased risk of acute myeloid leukaemia (AML) (Ades *et al*, 2014).

The link between immune dysregulation and MDS has been suggested (Glenthøj *et al*, 2016). Autoimmune attack to normal precursors as well as the activity of bystander T cells, recruited during an immune-response against dysplastic antigens, were hypothesised as relevant for the selection of dysplastic clones that are able to escape immune-mediated damage. The involvement of Natural Killer cells was also described (Terrazzano *et al*, 2012).

Basing on the evidence that innate and adaptive immune-effectors might participate in MDS development, several trials of immune-suppressive therapy have been performed. Younger age, Low Risk classification according to the International Prognostic Scoring System (IPSS) (Greenberg *et al*, 1997), the presence of the HLA-DR15 and high percentage of proliferating $CD4^+$ T cells have been considered as potential predictors of clinical response to immune-suppression (Sloand *et al*, 2008). However, valuable criteria to identify the subgroup of MDS patients susceptible to immune-modulating approaches, are currently lacking.

The T cell regulatory population (Tregs) are physiologically involved in the negative control of immune response (Sakaguchi, 2004). Increased Tregs in the late stages of MDS and the occurrence, in the first phases of the disease, of functional defects and altered migration patterns of this cell subset support the hypothesis that Tregs could play two opposite pathogenic roles in MDS (Kotsianidis *et al*, 2009; Alfinito *et al*, 2010).

Immune response has been fundamentally recognised as a finely tuned microsite process. Thus, the focus on BM immune profile represents a powerful tool for investigating Treg-mediated immune-tolerance control in the pathogenesis/progression of MDS. With this aim, we evaluated Tregs and cytotoxic T cells (CTL) in the BM of 37 MDS patients classified according to IPSS (Greenberg *et al*, 1997). The materials and methods are described in the online supporting information (Data S1). A significant increment ($P < 0{\cdot}05$) in the percentage of BM Tregs was observed in Intermediate-2 (Int-2)/High Risk patients in comparison with the Low/Int-1 groups (Fig 1A). Moreover, a reduction trend of CD54 expression, largely associated with the occurrence of antigen-dependent activation of CTL, was observed on BM-CTL from Low to the Int-2/High stages. BM CTL recruitment was then analysed by calculating the ratio between CTL percentage in the BM and peripheral blood (PB). There was a significant BM recruitment of CTL in Int-1 when compared with Low Risk group ($1{\cdot}74 \pm 0{\cdot}13$ vs. $1{\cdot}35 \pm 0{\cdot}08$; $P < 0{\cdot}05$), while reduced CTL recruitment in BM characterises the Int-2/High stage ($1{\cdot}07 \pm 0{\cdot}14$ in Int2/High vs. $1{\cdot}74 \pm 0{\cdot}13$ in Int-1; $P < 0{\cdot}05$) (Fig 1B, left).

We previously reported a clustered distribution of Tregs in BM of Low Risk patients and that a cut-off of 2% allows identification of two subgroups (Alfinito *et al*, 2010): thus, Low Risk patients were grouped accordingly (Fig 1B, right panel). As shown, the lowest (≤2%) Treg level was significantly associated with increased BM/PB CTL ratio ($1{\cdot}63 \pm 0{\cdot}14$ vs. $1{\cdot}21 \pm 0{\cdot}03$; $P < 0{\cdot}05$). No difference in BM recruitment of $CD4^+$ T cells was observed (not shown). Thus, BM-Treg level seems to preferentially control the BM recruitment of CTL in MDS.

Then, we analysed the cytotoxic T cells (TCR) repertoire in the PB and BM of healthy donors and MDS patients (Figure S1). $CD8^+$ and $CD4^+$ T lymphocytes were considered TCR-skewed when they expressed a single TCR-Vβ protein at a percentage higher than 3 standard deviations (SD) than observed in 10 healthy donors. Moreover, preferential BM-T cell expansions were defined as BM clones that showed a single TCR-Vβ protein expression exceeding the 20% of that observed in PB. Low Risk patients were then divided in two subgroups according to the number of observed BM-T cell expansions. The BM Treg percentage was significantly increased in Low Risk patients with <2 skewed Vβ families in BM CTL ($3{\cdot}04 \pm 0{\cdot}35$ vs. $1{\cdot}88 \pm 0{\cdot}58$; $P < 0{\cdot}005$; Fig 1C, left). Moreover, (Low Risk patients with ≥2 Vβ expansions in BM CTL showed significantly increased CD54 expression on CTL ($12{\cdot}99 \pm 2{\cdot}47$ vs. $3{\cdot}94 \pm 0{\cdot}42$; $P < 0{\cdot}05$; Fig 1D, left). No difference was observed when $CD4^+$ T cells were analysed (Fig 1C and D, right panels). Therefore, Tregs appear to exert a key role in the regulation of CTL activation/expansion in BM.

We previously observed that Tregs from Low Risk patients show a clustered, not homogeneous, distribution in BM and that a 2% cut-off value identifies two populations (Alfinito *et al*, 2010). Therefore, the occurrence of T cell expansions in BM of Low Risk individuals, categorised according to BM

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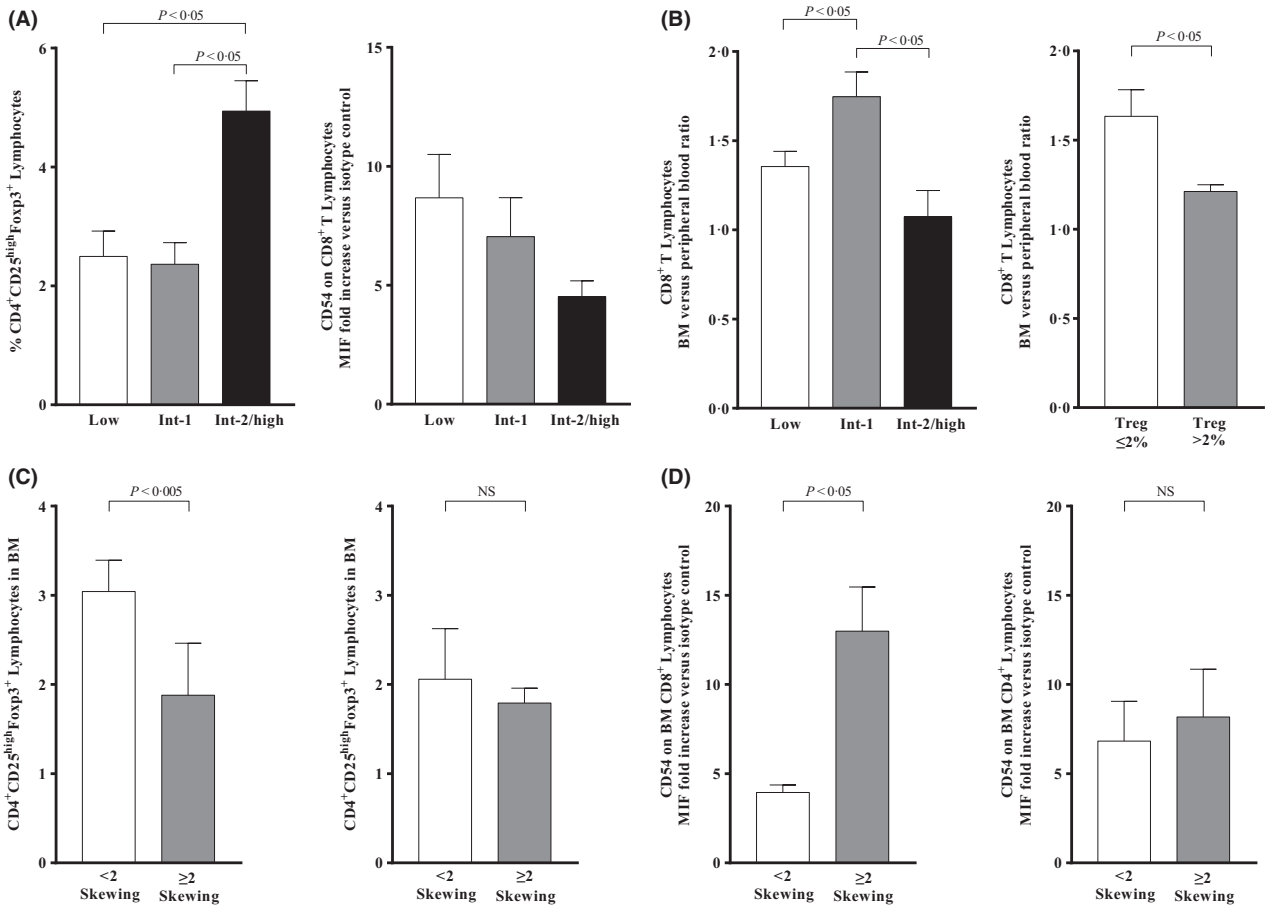

Fig 1. Treg level in BM preferentially controls BM-recruitment, activation and expansion of cytotoxic T cell effectors in Low risk MDS patients. (A) Treg percentage and CD54 expression on bone marrow cytotoxic T cells (BM-CTL) in myelodysplastic syndrome (MDS) patients classified as Low (white column), Intermediate 1 (Int-1; grey column) and Int-2/High (black column) risk, respectively; Tregs were significantly increased in Int-2/High, when compared to the Low ($P < 0{\cdot}05$) and Int-1 ($P < 0{\cdot}05$) individuals (left panel). For CD54 expression on BM-CTL, a trend of decreased expression was detected from Low and Int-1 to Int-2/High stages (right panel); (B) Left: there was a significant increase of BM-CTL in Int-1 ($P < 0{\cdot}05$), as compared with the Low Risk, while BM-CTL was decreased in Int-2/High Risk ($P < 0{\cdot}05$ as compared with the Int-1); White, grey and black columns indicate Low, Int-1 and Int-2/High risk patients, respectively; BM recruitment of CTL was evaluated by calculating the ratio between CTL percentage in BM and peripheral blood (PB); Right: BM recruitment of CTL in Low Risk patients categorised according to BM-Treg level [≤2% (white column) or >2% (grey column)], as described (Alfinito *et al*, 2010); Lower Treg percentage significantly correlated ($P < 0{\cdot}05$) with higher recruitment rate of CTL in BM; (C) Treg percentage and T-cell receptor (TCR)-Vβ skewing in BM-CTL and BM-$CD4^+$ T cells of Low Risk patients classified, according to the number of the T cell expansions, in two groups: <2 Vβ skewing (white column) vs. ≥2 Vβ skewing (grey column). An increase of Treg percentage in BM characterises Low risk patients with <2 TCR-Vβ skewed CTL, as compared those with ≥2 expansions in the BM (left); no significant difference was observed in the BM-Treg percentage when considering the $CD4^+$ T cell TCR-Vβ repertoire in the BM (right); (D) CD54 expression and TCR-Vβ skewing in BM-CTL and BM-$CD4^+$ T cells of Low Risk patients. As shown, individuals with ≥2 TCR-Vβ expansions in BM-CTL are characterised by significantly increased CD54 expression on CTL (left); no difference in CD54 expression was observed in $CD4^+$ T cells, despite the occurrence of a more skewed TCR-repertoire (right).

Treg level, was analysed. A significant increase of CTL expansions was detected in the BM of patients that showed lower (<2%) Treg level at disease onset ($P < 0{\cdot}05$; Table I). No significant association of Treg level with BM-$CD4^+$ T cell expansions was observed.

We also analysed leukaemia evolution and survival, in a minimal 36-month follow-up, in our Low Risk cohort grouped according to BM-Treg level. There was a significantly higher evolution to leukaemia ($P < 0{\cdot}05$) and death ($P < 0{\cdot}05$) in the Low Risk individuals with BM Tregs >2% at disease onset (Table I).

Taken together, our observations suggest that BM-Treg preferentially modulate CTL recruitment, activation and proliferation in BM of Low Risk MDS patients, and that their frequency at diagnosis inversely associates with an immune profile able to control disease progression.

A long-term follow-up study (Sloand *et al*, 2008) reported that responders to immune-suppression showed significantly longer survival with lower transformation to leukaemia. In addition, Tregs together with B cell progenitors were described as independent prognostic predictors in Low Risk patients, while overall survival and progression-free survival was

Table I. Follow-up evaluation of Low Risk myelodysplastic syndrome patients categorised according to regulatory T cell (Treg) level in the bone marrow (BM) at disease onset.*

	N	Age	CD8 skewed in BM ≥2	CD4 skewed in BM ≥2	Transfusion dependence	Leukaemiaevolution	Death
Low Risk	26	72·6 ± 9	13	10	8	5	6
BM Treg ≤2%	14	71 ± 5	10†	6‡	5	0§	0¶
BM Treg >2%	12	74·4 ± 11	3	4	3	5	6

*data refer to a minimum 36-month follow-up.
†significantly different from BM Treg >2% group ($P < 0·05$ by Fisher exact test; Odds Ratio (OR) 7·5 (95% confidence interval [CI]: 1·307–43·047).
‡not significantly different from BM Treg >2% group.
§significantly different from BM Treg >2% group ($P < 0·05$ by Fisher exact test); OR 0·047 (95% CI: 0·002279–0·9704).
¶significantly different from BM Treg >2% group ($P < 0·005$ by Fisher exact test); OR 0·034 (95% CI: 0·001679–0·7082).

significantly associated with lower Treg levels (Kahn *et al*, 2015). Moreover, the co-occurrence of MDS with autoimmune disorders was observed to predict longer survival and reduced leukaemia progression (Glenthøj *et al*, 2016; Komrokji *et al*, 2016). Accordingly, we found a significant association of lower Treg frequency and higher skewing in the Vβ BM-CTL TCR-repertoire, with decreased leukaemia progression and better overall survival in Low Risk MDS patients. Further investigation, addressing the molecular targets of BM skewed CTL, will hopefully clarify the role of immune-mediated processes in MDS pathogenesis and/or progression.

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Conflict of interest disclosure

The authors declare no competing financial interests.

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Supporting Information

Additional supporting information may be found online in the Supporting Information section at the end of the article.

Figure S1. Analysis of $CD4^+$ and $CD8^+$ T lymphocyte V-beta repertoire in BM and PB of healthy donors as compared with MDS patients.

Data S1. Methods and Materials.

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