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Short Communication

The bone marrow represents an enrichment site of specific T lymphocytes against filamentous fungi

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Abstract

Bone marrow has already been described as an enrichment site for several antigen-specific T lymphocytes, but the presence of mould-specific T cells has never been investigated in the bone marrow. We have previously demonstrated that mould-specific T cells emerge in the peripheral blood of patients with invasive fungal infections (IFI) but tend to become undetectable after disease resolution. In seven patients with a history of IFI, we investigated the presence of mould-specific T cells secreting different cytokines in bone marrow and peripheral blood paired samples. The results showed that the frequencies of mould-specific T cells secreting the protective cytokine IFNγ are significantly higher in bone marrow (BM) and are mainly represented by CD8+ T lymphocytes with effector phenotype. A putative disappearance of such protective BM responses after myeloablative therapy could contribute to the increased risk of IFI in hematologic patients.

Key words: Invasive Aspergillosis, Invasive Mucormycosis, antigen-specific T cells, bone marrow.

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Introduction

Invasive mould infections (IMI), mainly invasive aspergillosis (IA) and invasive mucormycosis (IM), remain a major cause of morbidity and mortality in patients with hematologic malignancies, in particular for acute leukemia patients and hematopoietic stem cell transplant (HSCT) recipients.[1] We previously demonstrated that, during the course of either IA or IM, mould-specific T cells may emerge in the peripheral blood (PB) of hematologic patients.[2–4] The detection of specific T cells against filamentous fungi in patients' PB not only represents an alternative diagnostic marker of IMI[5] but also seems to correlate with disease course. However, these mould-specific T cells tend to become undetectable in the PB after disease resolution.[3,4]

Different antigen-specific T cells have already been described to reside preferentially in the bone marrow (BM), namely, virus-specific[6–8] or tumor-specific T lymphocytes.[9–13] To address the hypothesis that BM could be a site of enrichment also for mould-specific T cells, we analyzed frequencies and phenotypes of mould-specific T-cell responses in paired BM and PB samples of hematologic patients with an history of IMI.

Material and methods

Patients

We studied a cohort of seven hematologic patients, who have developed IMI. According to EORTC/OMSG criteria,[14] four cases were classified as proven disease, namely, three IA and one IM, while the remaining three patients were classified as probable IA. Patients' clinical features are summarized in Table 1. Basal immunophenotypic analyses to assess absolute T-cell counts were routinely performed for clinical scope, during patients' follow-up. Paired samples of BM and PB were serially collected after IMI diagnosis (range 3–42 months) in order to perform mould-specific cytokine capture assays. Of note, in these sample series, median ratios of CD4/CD8 in BM (1.8 ± 0.7) and in PB (2.9 ± 1.3) were in line with normal values previously reported.[8,15] Written informed consents were obtained in accordance with the Declaration of Helsinki, after the local Ethical Committee's study approval (protocol no. 2414–63/11).

Methods

BM and PB mononuclear cells (BMMCs and PBMCs, respectively) were isolated by standard density gradient separation. The presence of mould-specific T cells was assessed by using the cytokine secretion assay (CSA) for different cytokines: interferon gamma (IFNγ), tumor necrosis factor alpha (TNFα), interleukin 10 (IL10), interleukin 4 (IL4), and interleukin 17A (IL17A). All experiments were conducted in triplicate according to the manufacturer's instructions, with few modifications, as already reported.[3,4] Briefly, 1×10^6 PBMCs or 1×10^6 BMMCs were stimulated with germinated and heat inactivated conidia, at a final concentrations of 100,000–200,000/ml, for 17 hours (IFNγ, TNFα and IL4 assays) or 40 hours (IL10 and IL17A assays). *Aspergillus conidia* or sonicated *Mucorales conidia* were used to detect reactive T cells in patients with either IA or IM, respectively. Unstimulated and PHA-stimulated PBMCs/BMMCs were used as negative and positive controls, respectively. Cells were acquired on a FACSCalibur flow cytometer and analyzed by using CellQuest and Summit softwares. The phenotype of the cytokine-producing cells was directly assessed after sample counterstaining with monoclonal antibodies directed to CD3 and to CD8 or CD4, and to the lymph node homing receptor CD62L (also CCR7 was used instead of CD62L in duplicated experiments). In addition, the cytotoxic phenotype of CD8+ or CD4+ T cells was assessed by using a monoclonal antibody against the degranulation marker CD107a.[13] The gating strategy is described in Supplementary Information S1 (Figure S1). Percentages of mould-specific cytokine+ T cells (out of either CD8+ or CD4+ T lymphocytes) were calculated as mean differences compared with unstimulated controls. In addition, the absolute frequencies of mould-specific IFNγ-secreting T cells were directly calculated from absolute counts of CD3+ CD8+ or CD3+ CD4+ cells (performed in parallel by using BD TruCOUNT$^{\text{TM}}$ tubes).

Statistical analysis

Statistical analysis were conducted using a Wilcoxon signed rank test to derive *P* values for comparing data between paired PB and BM samples; *P* values less than .05 were considered statistically significant.

Results

CSA analysis revealed the presence of mould-specific cytokine+ T cells in all the analyzed samples from all seven patients. At a median time of 12 months after IMI diagnosis (range 3–33), mean frequencies of these conidia-reactive T lymphocytes were generally higher in BM than in PB. Mean frequencies (percentage $\pm$ standard deviation) in BM, compared with PB, resulted as follows: IFNγ-producing T cells (**3.24%** $\pm$ **6.10** vs **0.67%** $\pm$ **1.24**), TNFα-producing T cells (**0.43%** $\pm$ **0.59** vs **0.37%** $\pm$ **0.64**), IL10-producing T cells (**0.22%** $\pm$ **0.33** vs **negative**), IL17A-producing T cells (**0.16%** $\pm$ **0.18** vs **0.15%** $\pm$ **0.23**) and IL4-producing T cells (**0.59%** $\pm$ **0.63** vs **1.27%** $\pm$ **1.35**) (Figure 1A). Of note, only the differences in IFNγ-producing T cells between BM

Table 1. Patients' clinical features.

Patient no.	Sex/Age (yr)	Underlying disorder or condition	Site of Infection	Isolates/biopsy	Galactomannan	Analyzed samples (months after IMI diagnosis)
1	F/27	AML	Lung/maxillary sinus	IA proven	pos	3
2	F/59	AML	Lung	IA proven	pos	8;12;24
3	F/40	AML	Lung	IA proven	neg	9;12;24
4	M/72	AML	Lung	*Aspergillus* spp/ IA probable	pos	4;6
5	F/47	AlloSCT	Lung	-/IA probable	pos	6;12;24
6	F/61	AlloSCT	Lung	-/IA probable	pos	12;24
7	M/40	AML	Left cavernous sinus	IM proven	neg	24;33;42

Note: F = female; M = male; AML = acute myeloid leukemia; AlloSCT = allogeneic stem cell transplant; IA = invasive aspergilosis; IM = invasive mucormycosis; pos = positive; neg = negative; IMI = invasive mould infection.
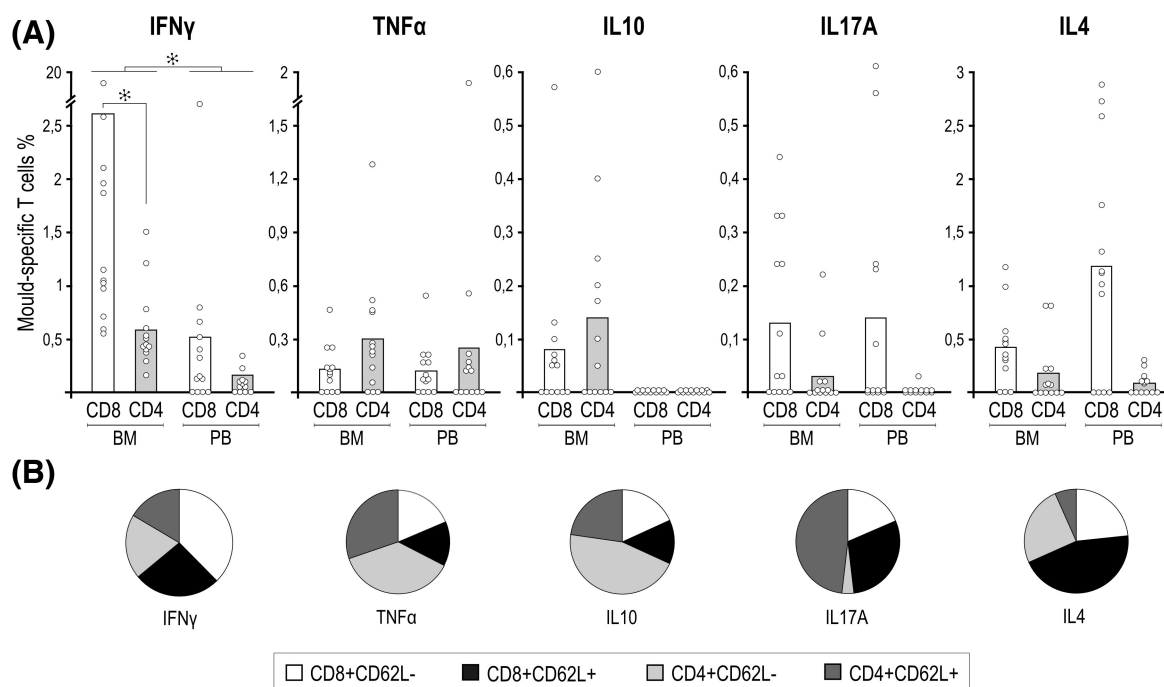

Figure 1. Characterization of mould-specific T cells in 7 patients with history of Invasive Mould Infection (IMI), by performing Cytokine Secretion Assays (CSA) on paired bone marrow (BM) and peripheral blood (PB) samples collected at median time of 12 months after IMI diagnosis. (A) Frequencies of mould-specific T cells secreting IFNγ, TNFα, IL10, IL17A or IL4, in BM and PB samples. Results are expressed as mean percentages of CD8+ T cells (white columns) or CD4+ T cells (gray columns). Magnitudes of specific cytokine+ T cells for individual time points are also reported in each columns (white circles). All patients showed higher proportions of mould-specific T cells in the BM compared to PB. Concerning to IFNγ-producing T cells, differences between BM and PB were statistically significant ($P = .0051$), as well as differences between CD8+ and CD4+ in the BM ($P = 0.0125$). *$P < .05$, Wilcoxon signed rank test. **(B)** Expression profiles of the homing lymphocyte receptor CD62L in mould-specific BM T-cell repertoire. For each cytokine+ mould-specific T-cell subset, white portions represent the mean percentages of CD8+ CD62L- T cells, black portions represent the mean percentages of CD8+ CD62L+ T cells, light gray portions represent the mean percentages of CD4+ CD62L- T cells, dark gray portions represent the mean percentages of mold-specific CD4+ CD62L+ T cells.

and PB were statistically significant ($P = .0051$). Moreover, among these specific IFNγ-secreting BM T lymphocytes, the fraction of CD8+ T cells (**2.65% $\pm$ 5.73**) was significantly higher than CD4+ T cells (**0.59% $\pm$ 0.45**) ($P = .0125$). The analysis of CD62L expression among mould-specific cytokine+ BM T cells is reported in Figure 1B. Concerning to IFNγ-producing subsets, we observed that both CD8+ and CD4+ were skewed to a CD62L- effector phenotype

(CD62L-/CD62L+ ratios were 1.42 and 1.18 in CD8+ or CD4+ T-cell subsets, respectively).

In 4 out of 7 patients, we had the possibility to longitudinally collect and test some further paired BM and PB samples, collected at a median time of 27 months after IMI diagnosis (range 24–42), aiming to investigate whether such protective BM immune responses may (i) persist after long periods since the complete resolution of IFI, (ii) modify their
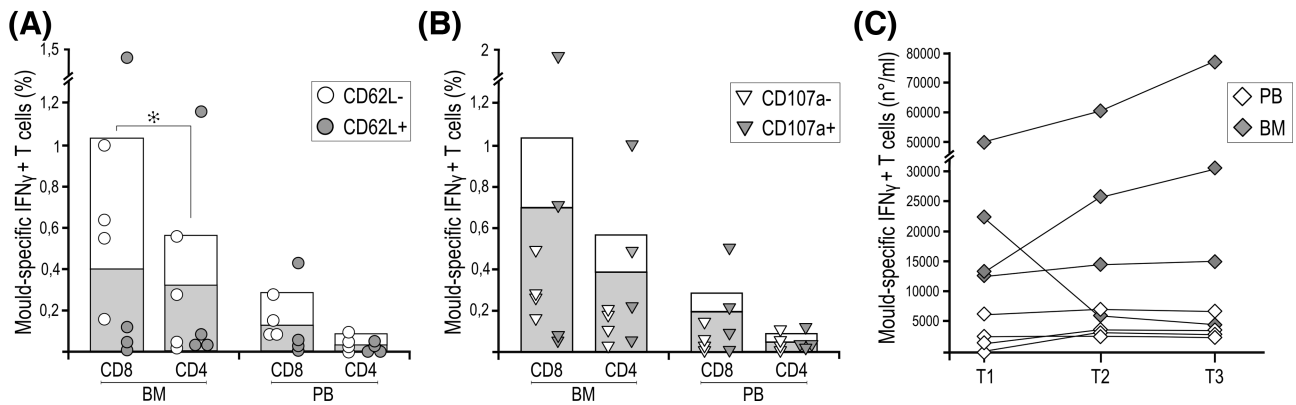
Figure 2. Characterization of IFNγ-producing mould-specific T cells in 4 out of 7 patients with history of IMI, by performing CSA analyses on paired BM and PB samples collected at median time of 27 months after IMI diagnosis. (A) The expression of CD62L among IFNγ-secreting mould-specific T cells in BM and PB samples is shown as mean percentage of CD62L- T cells (white columns) and CD62L+ T cells (gray columns), within either CD8+ or CD4+ T-cell subsets. In addition, magnitudes of single data points are depicted as CD62L- (white circles) and CD62L+ (gray circles). CSA analyses confirmed that mould-specific IFNγ-producing T cells were still prevalently enriched in the BM and that IFNγ was produced more frequently by CD8+ T cells than by CD4+ T cells ($P = 0.025$). *$P < .05$, Wilcoxon signed rank test. **(B)** The expression of the degranulation marker CD107a shows relevant proportions of CD8+ and CD4+ mould-specific IFNγ-producing BM T cells with potential cytotoxic activity, similar to what observed in PB samples. Gray and white columns represent the mean percentages of CD107a+ and CD107a-, respectively, within either CD8+ or CD4+ T-cell subsets, in BM and PB samples. In addition, magnitudes of single data points are depicted as CD107a+ (gray triangles) and CD1047a+ (white triangles). **(C)** Comparison of absolute numbers of IFNγ-producing mould-specific T cells over time, for the 4 patients with 3 time points analyzed. Gray and white diamonds represent data from BM and PB samples, respectively.

CD62L expression, and (iii) reveal cytotoxic potentials. Indeed, in this second set of experiments, we were able to confirm that mould-specific IFNγ-producing T cells were still prevalently enriched in the BM (BM **1.59%** ± 1.3 vs PB **0.34%** ± 0.16, $P = .017$) and that IFNγ was produced more frequently by CD8+ T cells (**1.02%** ± 0.84) than by CD4+ T cells (**0.57%** ± 0.46), in a statistical manner ($P = .025$) (Figure 2A). This consistent BM enrichment was also evident after calculation of the absolute numbers of BM and PB conidia-reactive IFNγ+ T cells over time (shown in Figure 2C).

When considering CD62L expression profiles among these long-lasting BM T lymphocytes, we observed that CD4+ T cells switched to a prevalent CD62L+ phenotype (CD62L-/CD62L+ ratio = 0.73), while CD8+ T cells did not show a relevant modification (CD62L-/CD62L+ ratio = 1.44) (Figure 2A). Furthermore, with regard to the surface expression of the degranulation marker CD107a, mould-specific IFNγ-producing BM T cells showed relevant proportions of CD8+ (mean **0.69%** ± 0.79) and CD4+ (mean **0.44%** ± 0.36) T cells with potential cytotoxic activity, similar to what observed in PB samples (Figure 2B).

Discussion

To our knowledge, this is the first report showing the presence of T-cell immune responses against filamentous fungi in the BM. We serially performed CSA immunoassays for different cytokines to identify and functionally character-

ize mould-specific CD8+ and CD4+ T cells in paired BM and PB samples, longitudinally collected from a series of leukemic patients, who developed IMI (either IA or IM) during induction chemotherapy or after HSCT. Here we demonstrated that 7 out of 7 patients (100%) showed significantly higher proportions of mould-specific T cells in the BM, compared with the PB. In particular, these specific BM immune responses were characterized by high frequencies of protective IFNγ-producing T cells, which still remained detectable several months after IFI complete regression.

These data evidenced that BM may play a pivotal role as a reservoir of mould-specific T cells in patients with a history of either IA or IM. This finding is in line with previous immunologic reports, which have described the BM as a relevant enrichment site for memory T lymphocytes,[16] against hematologic malignancies[9,11–12] and solid tumors,[10,13] as well as against different human viruses.[6–8] Of note, we showed that mould-specific IFNγ-producing BM T cells were mainly "effector" CD62L- CD8+ T-cells, and this resembles the prevalent protective BM T-cell subset with a memory role against WT1,[13] BCR-ABL1,[11–12] EBV[6–7] or CMV[6,9] antigens. Moreover, we observed a late switch toward "central" CD62L+ CD4+ IFNγ+ T cells, while no relevant changes occurred among CD8+ subsets. This late profile of mould-specific T cells is similar to the mixed patterns of effector memory (EM) CD8+ and central memory (CM) CD4+ specific T cells, detected in the BM of EBV+ healthy subjects[7] and in the BM of leukemic patients controlling the disease.[11–12] However, with our experimental approach, we could not formally exclude that a small

subset within the pool of responding T cells was constituted by CD62L+ (CD45RA+) naive T cells.[17]

Furthermore, similarly to what reported for tumor-specific and virus-specific immune responses,[7,13] we also demonstrated the cytotoxic potentials of mould-specific IFNγ-producing BM T lymphocytes. At the opposite of mould-reactive IFNγ-secreting T lymphocytes, the frequencies of specific IL4+ T cells were markedly higher (even if not in a statistical manner) in the PB rather than in the BM. This observation is consistent with the "suppressive" role of IL4-producing Th2 cells, as well as with the notion about the plasticity of functional T-cell subsets, suggesting that Th1 memory cells may also co-express IFNγ and IL-4 to control secondary immune responses.[18,19]

Interestingly, Okhrimenko et al.,[8] suggested that, in healthy subjects, the BM is not the preferred site of homing for CD4+ memory T cells against *Candida albicans* (MP65 antigen), possibly because *Candida* is a nonfilamentous fungus (yeast) typically affecting skin and mucosa in immunocompetent patients. Our survey has shown for the first time that, against filamentous fungi, long-lasting immune responses (mainly constituted by specific IFNγ-producing T lymphocytes) are markedly enriched in the BM of hematologic patients who previously developed angioinvasive fungal diseases. Additional analyses are warranted to describe functional EM/CM T-cell subsets within mould-specific BM repertoire, including double-cytokine+ subsets and different specificities to conidia-derived antigens.

In conclusion, a likely scenario is that long-term IFNγ-producing ("protective") T cells against moulds may preferentially rest in the BM and move to the PB in response to antigenic rechallenges. Intensive cytotoxic chemotherapy (i.e., myeloablative CHT, used as conditioning treatment for HSCT and as induction therapy for acute leukemias) is typically able to eliminate the majority of T cells residing in the BM. Thus, it is possible to speculate that the impairment of these mould-reactive BM T lymphocytes due to myeloablative CHT could represent a relevant factor contributing to the increased risk of IMI in HSCT and acute leukemia patients. Further studies are required to investigate whether such anti-fungal immune reservoir in the BM may have an essential protective role in the prevention of IMI. In perspective, BM may be exploited as a source for expansion of A*spergillus*-specific and *Mucorales*-specific T cells, in order to plan adoptive therapeutic strategies in IMI patients candidate to HSCT.

Author contributions

Conceived and designed the experiments: DV, GR, PBa, and LP. Performed the experiments: DV, GR, IL, and CQ.

Analyzed the data: DV, GR. Wrote the paper: DV, GR and LP. Provided well-characterized patient samples and related data: FF, MMo, PBr, AC, VC, MMa, AP, EC, RM, FN, PC, DC and TT. Critically revised the manuscript: FF, JPL, LR, PC, DC, TT, and ML.

Declaration of interest

M. Luppi serves in Advisory Boards for Merck Sharp & Dohme and Gilead Sciences, and received honoraria from these two pharmaceutical industries and from Pfizer and Nanogen. L. Potenza serves in an Advisory Board for Merck Sharp & Dohme and received grant support from Pfeizer. M. Luppi, L. Potenza and P. Barozzi have applied for an Italian patent regarding clinical applications of the ELISpot assay for the diagnosis of *Aspergillus* infection [PCT: WO2008/075395A3, EP2094295, IT2007/000867]. M. Luppi, L. Potenza, D. Vallerini, P. Barozzi and F. Forghieri have applied for an European and US patent regarding clinical applications of the ELISpot assay for the diagnosis of Mucorales infection (number MI2010A002224). This does not alter the authors' adherence to all Journal policies.

Supplementary Material

Supplementary material is available at *Medical Mycology* online (http://www.mmy.oxfordjournals.org/).

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