Activation of cytotoxic T-cell receptor γδ T lymphocytes in response to specific stimulation in myelodysplastic syndromes

Jean-Jacques Kiladjian,[1,2] Géraldine Visentin,[1] Emilie Viey,[1] Sylvie Chevret,[1] Virginie Eclache,[4] Jerome Stirnemann,[5] Jean Henri Bourhis,[1,6] Salem Chouaib,[3] Pierre Fenaux,[2] and Anne Caignard[1]

[1]INSERM U753, Institut Gustave Roussy, Villejuif; [2]AP-HP, Hôpital Avicenne, Service d'Hématologie Clinique, Bobigny, and Université Paris 13; [3]APHP, Hôpital Saint-Louis, DBIM, Paris; [4]APHP, Hôpital Avicenne, Laboratoire d'Hématologie, Bobigny; [5]APHP, Hôpital Jean Verdier, Service de Medecine Interne, Bondy; [6]Institut Gustave Roussy, Service d'Hématologie, Villejuif, France

J-JK and GV contributed equally to these studies.

Acknowledgments: we thank Robin Dhote, Géraldine Falgarone, and Claire Larroche for inclusion of patients with autoimmune disorders in the study.

Funding: this work was supported by INSERM, and Fondation de France (Comité Leucémies). GV was supported by the "Association Cent pour Sang la Vie" and AMGEN. BrHPP molecule (Phosphostim™) was obtained from Innate Pharma, Marseille, France.

Manuscript received June 5, 2007. Manuscript accepted November 28, 2007.

Correspondence:
Anne Caignard, INSERM U753, Institut Gustave Roussy, 39 rue Camille Desmoulins, Villejuif, 94805, France. E-mail: caignard@igr.fr

The online version of this article contains a supplemental appendix.

ABSTRACT

Background
We previously reported that the function and proliferation of natural killer cells in myelodysplastic syndromes are defective. T-cell receptor γδ T cells are other important components of innate immunity that have been recently implicated in the immune response against hematologic malignancies.

Design and Methods
We evaluated the phenotype, function, and *in vitro* expansion of myelodysplastic syndrome patient-derived γδ T cells in response to interleukin-2 and bromohalohydrin pyrophosphate, a synthetic phosphoantigen with a potent T-cell receptor γδ agonist effect that specifically activates and amplifies this T-cell population.

Results
Vγ9Vδ2 T cells, the major circulating γδ T-cell subset, were reduced in myelodysplastic syndromes, but mainly in myelodysplastic syndromes' patients with associated autoimmune diseases, suggesting that this anomaly was largely due to the autoimmune component. On the other hand, bromohalohydrin pyrophosphate-induced expansion of the Vγ9Vδ2 T-cell population in all 15 control samples, but in only 26 of 43 (60%) myelodysplastic syndromes patients. The response to bromohalohydrin pyrophosphate was independent of World Health Organization subtype, cytogenetic findings and International Prognostic Scoring System score. In responding myelodysplastic syndromes patients, expanded Vγ9Vδ2 T cells exhibited normal cytolytic and secretory activity against leukemic and myelodysplastic syndromes cell lines; fluorescence *in situ* hybridization analysis indicated that these Vγ9Vδ2 T cells were not derived from the myelodysplastic syndromes clone. However, these Vγ9Vδ2 T cells from the MDS patients had limited proliferative capacity in response to interleukin-2 despite having normal expression of interleukin-2 receptor chains (αβγ).

Conclusions
These results, combined with our previous findings concerning natural killer cells, suggest that there are immune surveillance defects in myelodysplastic syndromes, which may contribute to the pathogenesis of these syndromes.

Key words: innate immunity, myelodysplasia, cellular immunology.

Citation: Kiladjian J-J, Visentin G, Viey E, Chevret S, Eclache V, Stirnemann J, Bourhis JH, Chouaib S, Fenaux P, and Caignard A. Activation of cytotoxic T-cell receptor γδ T lymphocytes in response to specific stimulation in myelodysplastic syndromes. Haematologica 2008 Mar; 93(3):381-389. doi: 10.3324/haematol.11812

Introduction

Myelodysplastic syndromes (MDS) are clonal hematopoietic stem cell disorders characterized by ineffective hematopoiesis and frequent progression to acute myeloid leukemia. The pathophysiology of these syndromes remains poorly explained.[1] Several immune defects, including hypo- or hyper-gammaglobulinemia, peripheral lymphopenia, abnormal B or T-cell functions, monoclonal gammopathy, auto-antibodies and overt autoimmune disorders have been shown to be present at higher than expected incidences in patients with MDS.[2-4] The immune system also seems to contribute to the progressive cytopenias observed in MDS in some cases. Indeed, the higher level of apoptosis reported in MDS bone marrow myeloid progenitors can be triggered by pro-inflammatory cytokines released by macrophages and/or lymphoid T cells in the microenvironment.[5] Furthermore, immunosuppressive therapy has been reported to improve peripheral cytopenias in some cases of MDS.[6]

The possible use of innate effector lymphocytes, including natural killer (NK) cells, NKT cells and γδ T cells, for tumor immunotherapy is of recent interest.[7,8] These effector cells detect altered self or stress molecules expressed by various transformed cells, which are then killed through cell-mediated lysis. We recently demonstrated the existence of several NK cell defects in MDS, including weak lytic and secretory capacities, and an absence of proliferation *in vitro*.[9]

T-cell receptor γδ T cells constitute an important subset of cytotoxic non-conventional effectors involved in immune reactions against mycobacteria and tumors. T lymphocytes bearing a γδ-T cell-receptor, formed by the Vγ9Vδ2 rearrangement, constitute a minor subset of human peripheral T cells (1-10%). The number of these cells in the blood increases with age, suggesting positive selection in the periphery due to antigenic stimulation.[10] This cell population has lytic potential against various tumor cell lines *in vitro*[11-13] and could play a role in clinical responses in lymphoid malignancies including lymphoma and multiple myeloma.[14-16] In addition, γδ T cells are potent and rapid producers of interferon-γ and tumor necrosis factor-α,[17] and potentially act as antigen-presenting cells.[18] They have a regulatory function, interact with myeloid cells and are induced in various forms of tolerance.[19,20]

Several small, phosphorylated, non-peptide metabolites synthesized by *Mycobacterium tuberculosis* have been found to stimulate Vγ9Vδ2 T cells *in vitro*.[21,22] The most potent of these is isopentenylpyrophosphate.[23] We previously showed that a synthetic analog of isopentenylpyrophosphate, bromohalohydrin pyrophosphate (BrHPP, Phosphostim™) induced a large *in vitro* expansion of Vγ9Vδ2 T cells derived from peripheral blood mononuclear cells (PBMC) from healthy donors and patients with metastatic renal cell carcinoma.[24]

In this study, we investigated γδ T cells in MDS in order to gain further insights into the mechanisms of development of immune abnormalities in MDS patients, and to assess the role of these cells as cytotoxic effectors potentially useful for autologous or allogeneic cellular therapy.

Design and Methods

Patients and cell cultures

Thirty healthy donors and 44 MDS patients from four centers of the *Groupe Français des Myelodysplasies* were included in this study. Institutional Review Board approval for these studies was obtained from the Groupe Français des Myelodysplasies Ethics Committee, and all patients signed an informed consent form. Ten MDS patients had concomitant autoimmune disorders (AID), including rheumatoid arthritis (n=5), thyroiditis (n=1), systemic lupus erythematosus (n=1), antiphospholipid syndrome (n=1) and auto-antibodies (n=2). Given the high incidence of associated AID in this cohort of MDS patients, a series of 12 consecutive unselected non-MDS patients with AID were enrolled as controls. These patients (six with rheumatoid arthritis, five with systemic lupus erythematosus, and one with several types of auto-antibodies) were recruited from two Departments of Internal Medicine after having given informed consent to inclusion in the study. At the time of blood sampling all these patients had active disease; seven newly diagnosed patients were not receiving any immunomodulatory treatment, two were receiving corticosteroids and methotrexate, and three were being treated with infliximab. Their median age (60.5 years; range: 19-88) was lower than that of the MDS patients (median 69.6 years; range, 45 to 85), and there was a female predominance (66% females in the control AID group vs 45% in the MDS group), but these differences were not statistically significant (p=0.1, and p=0.43, respectively).

Blood samples (15-30 mL) were collected and PBMC were isolated by centrifugation on a Ficoll-Hypaque density gradient (Amersham Biosciences). PBMC from healthy donors were treated in parallel with patients' samples to control for γδ amplification. Between $2x10^6$ and $10x10^6$ PBMC were cultured at a density of $2x10^6$/mL in 500 μL of RPMI 1640 supplemented with 10% v/v fetal calf serum (Fetal Clone irradiated, HyClone) in a 24-well plate. BrHPP (Innate Pharma, Marseille, France) was added once, to a final concentration of 3 μM at the start of the culture, together with 100 IU/mL interleukin(IL)-2 (Aventis Pharma, Romainville, France). On day 5 and every 3 days thereafter, the medium containing IL-2 (100 IU/mL) was replaced. In some experiments, IL-2 was replaced by IL-15 (20 ng/mL) or used in combination with IL-7 (10 ng/mL) or IL-21 (40 ng/mL). The expansion of γδ T cells was determined on days 5, 8, 11 and 14 by flow cytometric assessment of the percentages of CD3Vδ2 T cells.

^{51}Chromium release assay, CD107a mobilization test and interferon-γ secretion test

^{51}Cr release assay. When the proportion of Vδ2 T cells generated by BrHPP/IL-2-induced stimulation of MDS patients' PBMC exceeded 85%, the cytotoxicity of the expanded-Vγ9Vδ2 T cells was determined in a 4 h ^{51}Cr release assay using the sensitive cell lines Daudi and P39, and the resistant cell line Raji. These target cell lines were labeled with 100 μCi ^{51}Cr for 60 min. The effector to target ratio ranged from 40:1 to 5:1. Specific lysis (expressed as a percentage) was calculated using the standard formula [(experimental-spontaneous release/total-spontaneous release) x 100]; the mean of the triplicates was recorded. Lysis values for Vγ9Vδ2 T cells from donors and MDS patients were compared using Student's t-test.

CD107a mobilization test. The lytic capacity of expanded Vδ2T cells was assessed using the CD107a mobilization test (CD107a-FITC, Immunotech). This test allows the analysis of a T-cell subset of interest in bulk populations. The degranulation potential of Vδ2 T cells was measured using P815 FcR-positive cells coated with anti-human T-cell receptor γδ monoclonal antibody (IMMU 510, Beckman Coulter) to trigger T-cell receptors.

Secretion of interferon-γ by activated Vδ2T cells: Br-HPP activated Vδ2T cell cultures from donors and MDS patients were incubated for 6 h in 96-well plates coated with anti-human T-cell receptor γδ monoclonal antibody; supernatants were harvested and interferon-γ secretion was measured by an enzyme linked immunosorbent assay (BD OptEIA Set Human IFNγ, BD Sciences).

Expression of IL-2 receptor chains

PBMC were treated with BrHPP (3 mM) and IL-2 (100 IU/mL) for 5 to 8 days. The expression of IL-2 and IL-15 receptor chains was assessed by flow cytometry on Vδ2 T cells *ex vivo* and on Vδ2 T cells activated by BrHPP+IL-2. Alternatively, PBMC were stimulated for 2 to 5 days with 100-500 IU/mL IL-2 (or IL-15) and IL-2Rα expression was analyzed. Membrane expression of IL-2Rβ chain (CD122), IL-2Rγ chain (CD132) and IL-2Rα chain (CD25) on CD3$^+$, CD3$^+$Vδ2 and CD3$^-$CD56$^+$ NK cells was determined using conjugated monoclonal antibodies.

Fluorescence in situ hybridization (FISH)

Immunoselected T-cell receptor γδ T cells expanded from MDS patient-derived PBMC were allowed to adhere to polylysine™ glass slides (O Kindler GmbH, Freiburg, Germany) and fixed in methanol/acetic acid (3:1). Interphase FISH was performed with the following probes, according to the manufacturer's instructions: a spectrum orange- and fluorescein-labeled dual probe (D7Z1/D8Z1) for simultaneous detection of the chromosome 7 and 8 centromeres; spectrum orange and spectrum green-labeled probes (LSI CSF1R /D5S23-D5S721) for simultaneous detection of the chromosome 5 regions 5q33 and 5p15.2 (all from Abott, Rungis, France). Cell nuclei were counterstained with DAPI (Vectashield, Vector Laboratories Inc., Burlingame, USA). Fluorescent signals were viewed with a Zeiss Axiophot epifluorescence microscope (Carl Zeiss SA, Le Pecq) linked to MetaSystems software (Altlussheim, Germany). Between 50 and 200 cells were counted for each specimen by independent observation.

Results

Clinical and hematologic characteristics of the patients

The 44 unselected consecutive MDS patients enrolled in the study comprised five with refractory anemia/refractary anemia with ringed sideroblasts, 13 with refractory anemia with excess of blasts (RAEB)-1, ten with RAEB-2, nine with refractory cytopenia with multilineage dysplasia, two with unclassifiable MDS, four with MDS with isolated del(5q), and one with MDS-myeloproliferative disorder, according to the WHO classification. None had previously received cytotoxic or immunomodulatory treatment. The clinical and hematologic characteristics of these patients are summarized in *Online Supplementary Table 1*. Their median age was 69.6 years (range, 45 to 85), and the median follow-up since the diagnosis of MDS was 14 months (range, 0 to 169). The International Prognostic Scoring System (IPSS) score,[25] available for 36 patients with successful cytogenetic analysis, was low in six, low intermediate (INT-1) in 18, high intermediate (INT-2) in eight, and high in four of the patients. Eight patients developed acute myeloid leukemia during the follow-up, and ten patients presented concomitant AID. MDS patients with or without AID had similar age (p=0.43), sex ratio (p=0.47), white cell count (p=0.82), polymorphonuclear cell count (p=0.33), platelet count (p=0.38), distribution of MDS subtypes (p=0.17), IPSS score (p=0.62), progression to acute myeloid leukemia (p=0.38), and survival (p=0.24). However, MDS patients with associated AID had lower hemoglobin levels (median 8.45 vs 9.7 g/dL; p=0.01) and longer follow-up since the diagnosis of MDS (median 54 vs 13 months; p=0.014) than MDS patients without AID.

The percentage of peripheral Vδ2 T lymphocytes is lower in MDS with associated AID

Before *in vitro* stimulation, the percentages of T cells that were CD3$^+$, CD4$^+$ and CD8$^+$ in MDS patients were in the same ranges as those of normal donors (13%-60.8% for CD8 T cells). The percentage of circulating Vδ2T cells, assessed using the CD3/TCRVδ2 combination, was significantly lower in MDS patients (median, 1.16%; range, 0.2-2.9%) than in healthy donors (median, 2%; range, 1.3-4%) (p=0.002). This percentage was significantly lower in MDS patients with concomitant AID (median, 0.2%; range, 0.06-0.48%) than in healthy donors (p=6x 10^{-4}), but was not significantly lower in

MDS patients without AID (median, 1.2%; range, 0.2-2.9%; p=0.10) (Figure 1). The difference in the proportions of circulating Vδ2T cells was, therefore, associated with the presence of AID. In 12 non-MDS patients with AID, the percentage of circulating Vδ2T cells was significantly lower (median, 0.37%; range, 0.17-0.95%) than in controls (p=0.002), and similar to that found in MDS patients with associated AID (p=0.41) (Figure 1). These differences in percentages of circulating Vγ9Vδ2 T cells were independent of age (p=0.35).

Response to stimulation with BrHPP combined with IL-2

PBMC from 43 MDS patients and 15 healthy donors (controls for γδ expansion), were cultured with BrHPP (3 μM) and IL-2 (100 U/mL) for 8 to 18 days. Consistent with previous reports,[24, 26] the percentage of Vδ2 T cells gradually increased in *in vitro* PBMC cultures from controls, accompanied by an exponential proliferation of the responding cells (>70% of Vδ2 T cells at 14-18 days, defining *high* responders) in all cases (Figure 2A).

A response to BrHPP stimulation was obtained in 60% of MDS patients. In detail, 12 of the 43 (28%) cultures displayed high Vδ2 T-cell expansion generating > 70% of Vδ2 T cells, 14 (32%) displayed intermediate expansion (between 30 and 70%), and 17 (40%) did not respond to BrHPP (< 30% Vδ2 T cells). Most MDS patients who did not respond to BrHPP had associated AID (7 of 10 patients, 70%) (Figure 2B). Of the 33 MDS patients without AID, 11 (33%) did not respond to BrHPP and IL-2; 12 were high responders, and ten cultures displayed intermediate expansion (Figure 2C). Stimulation with IL-2 alone did not induce expansion of the Vδ2 T-cell population. BrHPP responses were not correlated with age (p=0.58), MDS subtype (p=0.5), cytogenetic findings (p=0.3), or IPSS score (p=0.7). The lower percentage of circulating γδ T cells did not account for the different responses to BrHPP in MDS patients; indeed, a large expansion (>70%) of Vδ2 T cells occurred in some patients with very low percentages of circulating γδ T cells. Differing responses to BrHPP and IL-2 induced *in vitro* were observed in MDS patients with associated AID and in non-MDS AID patients; no expansion of the Vδ2 T cell population was observed in nine of the 12 (75%) patients analyzed (Figure 2D), suggesting that the differences in response to BrHPP *in vitro* were related to the presence of AID rather than MDS.

Subsequent studies were performed only on cells from MDS patients without AID, because the numbers of circulating Vδ2 T cells and their expansion rates were too low in MDS patients with AID for phenotypic or functional analyses.

Since the response to BrHPP is related to the differentiation status of the γδ T cells, we studied the Vγ9Vδ2 T-cell repertoire in PBMC from 12 MDS patients and ten healthy volunteers *ex vivo*. In humans, γδ T cells are classified into four subsets according to the expression pattern of CD45RA and CD27 markers: *naive* (T_N, $CD45RA^+CD27^+$) and *central memory* (T_{CM}, $CD45RA^-CD27^+$) γδT cells display high proliferation capacity but low effector function, whereas the reverse is true for *effector* (T_{EM}, $CD45RA^-CD27^-$) and *late effector* (T_{EMRA}, $CD45RA^+CD27^-$) γδ T cells.[27] In line with previous findings,[28] T_{CM} cells constituted the major subpopulation among circulating Vγ9Vδ2 T cells in most (8/10) of the healthy donors; T_{EM} cells were dominant in the other two healthy donors. A much more heterogeneous distribution of the different γδ-T cell subsets was found in MDS patients. T_{CM} cells were the predominant subpopulation in only six of the 12 MDS patients, whereas T_{EMRA} cells were predominant in four patients. Unexpectedly, two MDS patients had a predominant population of *naive* γδ T cells. Cultures (which could be performed in nine of these 12 MDS patients with sufficient numbers of γδ T cells for both analyses) resulted in expansion of the γδ T-cell population in all but one case, regardless of the size of the T_{CM} population; thus the skewed repertoire of γδ T cells in these MDS patients was not responsible for the altered T-cell receptor agonist-induced responses. These repertoire analyses were performed only in samples with >1% of γδ T cells (see *Design and Methods*), and the results may, therefore, be relevant only in patients in whom γδ T cells could be expanded *in vitro*.

In vitro analysis of clonal expansion and phenotype of expanded MDS-Vδ2 T cells

The BrHPP-stimulated-Vγ9Vδ2 T cells from 12 responding MDS patients were further phenotyped from days 8 to 12 of culture, using the panel of monoclonal antibodies described above. *In vitro* BrHPP-stimulation from normal PBMC induced the rapid proliferation of T_{EM} cells which became the major Vγ9Vδ2 T-cell subset by day 5 of culture. In MDS patients, all expanded Vγ9Vδ2 T lymphocytes had the same differentiation status (*data not shown*). Furthermore, these cells were activated ($CD69^+$) and expressed the lysis-triggering receptor NKG2D (median, 88.9%; range, 67.2-99.9%). A lower proportion of $CD8^+$ Vδ2T cells (5.2% versus 18.5%) and weaker CD8 expression, as assessed by median or mean fluorescence intensity (*data not shown*), were found in expanded Vδ2 T cells from MDS patients than in those from healthy donors. This suggests that different subsets of Vδ2 T cells were expanded in MDS patients and controls.

FISH analyses were performed in expanded Vδ2 T cells, purified by FACS, derived from six informative MDS patients with 5q- (n=4), +8 (n=1), or 11q- (n=1) chromosomal abnormalities. No chromosomal abnormality was detected in the Vδ2 T cells from these patients (*not shown*).

Functional activities are maintained in Vδ2 T cells expanded from MDS patients

The cytolytic potential of expanded Vδ2 T cells from MDS patients was assessed using two complementary assays. In cultures generating more than 85% Vδ2 T cells,

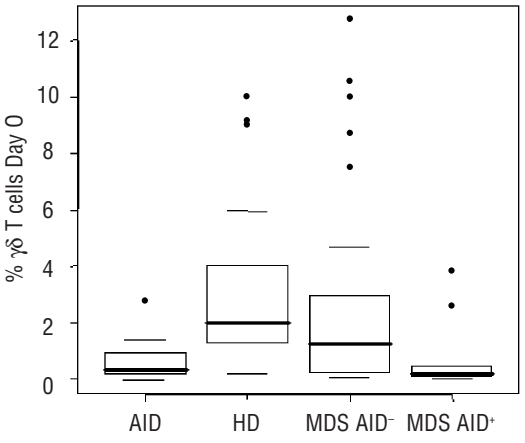

Figure 1. Percentages of peripheral Vδ2 T cells in healthy donors (HD, n=15), non-MDS patients with auto-immune disease (AID, n=10), MDS patients with no associated AID (MDS/AID⁻, n=32), and MDS patients with AID (MDS/AID⁺, n=10). Boxplots of Vδ2 T-cell percentages on day 0 from the four populations analyzed show a lower proportion of Vδ2 T cells in AID⁺ and MDS/AID⁺ patients than in MDS/AID⁻ and HD.

cell-mediated lysis was measured by the ^{51}Cr release assay. The Burkitt's lymphoma cell lines Daudi and Raji were used as sensitive and resistant targets, respectively, to assess γδ-mediated lysis, and the P39 cell line was used as a representative MDS target. In all eight MDS-derived Vδ2 T-cell cultures, the percentage lysis for the Daudi cell line was similar to that obtained for donor Vδ2T cell cultures (n=10). The MDS cell line, P39, was efficiently killed by both MDS- and donor-derived activated Vδ2 T cells (Figure 3A).

The externalization of lysosome associated membrane protein (LAMP-1 or CD107a) by activated T and NK cells following stimulation by a target provides an alternative way to measure cell-mediated lysis by T or NK cell subsets without their prior immunoselection.[29] Expanded T cells were specifically stimulated with T-cell receptor γδ monoclonal antibody-coated P815 target cells and degranulation was measured by CD107a staining. The level of γδ T-cell degranulation was similar between donors and responding MDS patients (Figure 3B). In Vδ2 T cells derived from six *intermediate* responders (i.e. cultures of cells from patients containing 30 to 70% Vδ2), degranulation of MDS-Vδ2 T cells was similar to that of donor-derived cells (Figure 3C); thus, even in patients with intermediate levels of expansion, γδ T cells expanded *ex vivo* were lytic. In addition, T-cell receptor agonist and IL-2 induced the secretion of interferon-γ by Vδ2 T cells. Large amounts of interferon-γ (>4 ng/mL) were secreted by both MDS patient-derived (n=8) and donor-derived (n=10) Vδ2 T cells following T-cell receptor triggering. Thus, Vδ2 T cells expanded from MDS patients are lytic and can secrete cytokines, like normal Vδ2 T cells.

Vδ2 T cells expanded from MDS patients have weak IL-2 induced proliferation

γδ T-cell cultures from responding MDS patients displayed a weaker proliferative response to IL-2 than donor-derived cultures, despite an efficient initial expansion of Vδ2 T cells. The amplification index (ratio of absolute numbers of Vδ2 T cells on day 14 divided by the absolute numbers of Vδ2 T cells on day 0) was determined in ten cultures from MDS patients containing >70% Vδ2T cells and in 15 control cultures derived from healthy donors. The mean amplification index in MDS patient-derived cells was 93 (range, 14 to 191), whereas it was 389 (range, 90 to 950) in controls (p=0.0023) (Figure 4A, left panel). Thus, although Vδ2 T cells from MDS patients were expanded with a similar efficiency to that of cells from healthy donors, they displayed a lower capacity to proliferate *in vitro* in response to IL-2. MDS patients responding to BrHPP had a higher percentage of

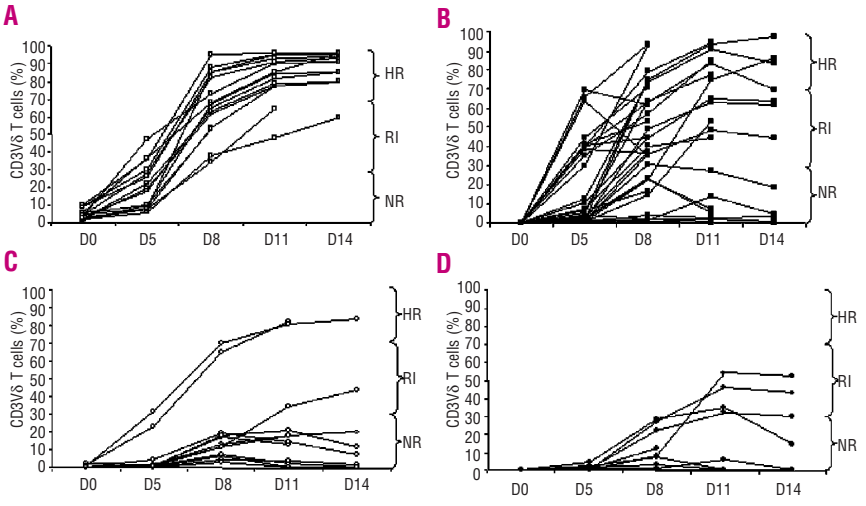

Figure 2. Response to *in vitro* activation of T-cell receptor Vδ2T cells from donors, MDS patients with or without AID, and AID patients. PBMC (2x10⁶ cells/mL) from 15 healthy donors (HD: panel A), 43 MDS patients (panels B and C) and 12 non-MDS AID patients (panel D) were simulated with BrHPP (3 μM) and IL-2 (200 IU/mL). Ten of the MDS patients had associated AID (panel C, n=10). PBMC (2x10⁶/mL) were cultured in RPMI 1640 supplemented with 10% v/v fetal calf serum in a 24-well plate. BrHPP was added once, to a final concentration of 3 μM at the start of the culture, together with 100 IU/mL IL-2. On day 5 and every 3 days thereafter, medium containing IL-2 (100 IU/mL) was replaced. Percentages of CD3Vδ2 T cells were determined on days (D) 0, 5, 8, 11 and 14 following stimulation.

Vδ2T cells than healthy volunteers (MDS patient median: 4.5% (range, 2.1-7.5), although this difference was not statistically significant (Figure 4A).

Treatment with higher concentrations of IL-2 (500 and 1000 U/mL) did not increase the proliferation index of MDS Vδ2 T cells (*data not shown*). Cells were treated with IL-15. This cytokine displays similar biological activities to IL-2 and is active on memory T cells. The IL-2 and IL-15 receptors have two signal transducing chains, Rβ and Rγ, in common. MDS patient-derived cultures (n=7) stimulated with IL-15 (20 ng/mL) did not have a greater response to BrHPP or a higher proliferation index than cultures treated with IL-2. We also treated cells with a combination of IL-2 and two cytokines signaling through the γ chain, IL-7 and IL-21. These cytokines have homeostatic effects on memory T cells and synergize with IL-2 to induce the proliferation of T and NK cells. The combination of IL-2 with IL-7 or IL-21 had no effect on the proliferation of MDS-Vδ2 T cells (*data not shown*). We studied the surface expression of the IL-2 receptor α, β and γ chains on MDS Vδ2 T cells by flow cytometry to exclude the possibility that changes in IL-2 receptor expression were responsible for the absence of proliferation. We found constitutive expression of IL-2/15 Rβ (CD122) and γc (CD132) and weak expression of IL-2Rα (CD25) on resting MDS Vδ2T cells, as in Vδ2T cells from healthy individuals (Figure 4B). Phosphoantigen activates γδ T cells inducing CD25, so CD25 expression was assessed following 5 days of BrHPP and IL-2 treatment. The induction of IL-2Rα expression was similar in MDS Vδ2 T cells (n=6) and control Vδ2T cells (n=6); expression of the IL-2Rβ and IL-2Rγ chains was not modulated by BrHPP activation (Figure 4B).

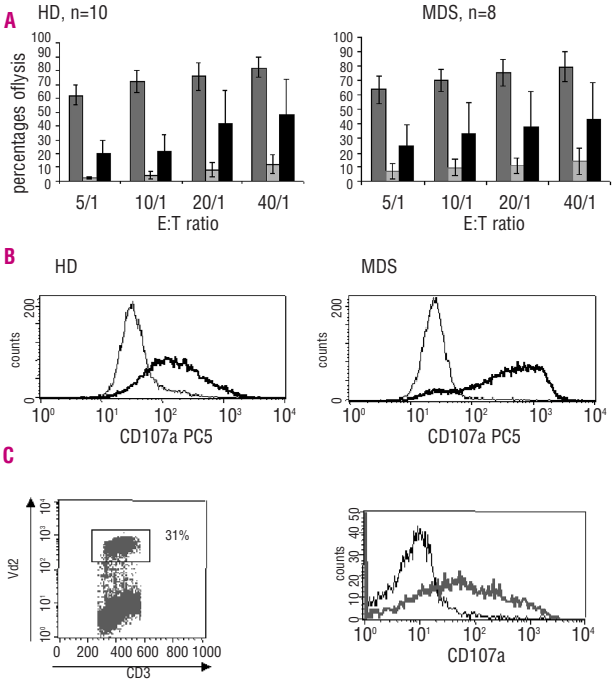

Figure 3. Functional analysis of BrHPP-expanded Vδ2 T cells from MDS patients. A. Vδ2T cells from MDS and donor PBMC were treated with BrHPP and IL-2 *in vitro* for 8 to 12 days. Cultures with >85% expanded Vδ2T cells were analyzed for ^{51}Cr release. The ^{51}Cr release assays were performed on Vδ2 T cells using Daudi (dark gray), Raji (light gray) and P39 (black) targets. Results are expressed as mean percentages from independent experiments on donors (n=10, left panel) and MDS patient-derived cultures (n=8, right panel). B. In addition, the lytic potential of γδ T cells was assessed by CD107a degranulation assays. Activated Vδ2 T cells from healthy donors (HD) and MDS patients were incubated for 3 h with T-cell receptor Vδ2-coated P815 cells and labeled with CD107a. One representative experiment of six is shown. C. CD107a degranulation of MDS patient PBMC-derived Vδ2 T-cell cultures generating 30 to 70% of Vδ2 T cells (left panel). Cells were incubated for 3 h with anti-T-cell receptor Vδ2-coated P815 cells, labeled with CD107a and stained with a PE-antiVδ2 monoclonal antibody. One representative experiment of six is shown.

Discussion

The benefits of allogeneic adoptive cellular therapy in MDS patients demonstrate the potential importance of immune cells in disease control.[30] We investigated the phenotype and function of T-cell receptor γδ T cells, an important component of innate immunity that has rarely been studied in MDS.[3132]

We found (i) a significantly lower baseline proportion and *in vitro* expansion of Vγ9Vδ2 T cells in MDS patients with concomitant AID, phenomena more likely related to the AID; (ii) a specific proliferation defect of MDS Vγ9Vδ2 T cells in response to IL-2; (iii) that it was possible to expand *ex vivo* phenotypically normal, fully functional, and non-clonal autologous Vγ9Vδ2 T cells in the majority of MDS patients.

The proportion of circulating Vγ9Vδ2 T cells was lower in MDS patients than in healthy donors. However, this was mostly attributable to MDS patients with associated AID, because MDS patients without AID had a similar percentage of circulating Vγ9Vδ2 T cells as controls. A control group of non-MDS patients with active AID had significantly lower percentages of Vγ9Vδ2 T cells than healthy controls, but a similar percentage to MDS patients with associated AID. Furthermore, comparable absence of expansion of γδ T cells in response to BrHPP was found in MDS and non-MDS patients with AID. These defects, therefore, seemed to be correlated to the existence of an associated AID.

It has been previously suggested that γδ T cells, particularly Vδ1 T cells, could play a role in the pathogenesis of AID.[19,33,34] Furthermore, Vδ2T cells are decreased in the peripheral blood of patients with lupus and arthritis, and could display regulatory properties in addition to the pathogenic contribution of Vδ1 T cells.[35] We found that the duration of MDS was significatly longer in those MDS patients with AID than in those without AID (median 54 vs 13 months; p=0.014). Such findings may suggest that, in a subset of MDS patients, altered proliferation of γδ T cells could progressively lead to a decrease in the circulating numbers of these cells, triggering subsequent development of AID. Alternatively, these results could suggest *in vivo* activation and deletion

of this T-cell subset in response to a particular environment in patients with AID, independently of MDS. Correlations between clinical or serological immunological abnormalities and other biological features have rarely been investigated in MDS. A recent retrospective study found that, apart from a female prevalence, the presence of AID was not correlated with any alterations concerning T, B or NK cells.[32]

Our study also provides new data on the physiology of γδ T cells in MDS. The repertoire of peripheral Vγ9Vδ2 T cells from healthy donors mostly consists of memory cells with a predominant subpopulation of T_{CM} cells. In MDS patients, the repertoire of Vγ9Vδ2 T cells was more heterogeneous, with a predominant T_{CM} population in only 50% of patients. A few patients exhibited an unusual repertoire with a large subset of naive cells or late effector memory cells. The small numbers of naive and central memory cells, and the predominance of effector memory cell subsets in MDS is, however, also consistent with previous findings of differences in γδ repertoire changes between young adults and older subjects and could reflect the older median age of the MDS patients.[36] Alternatively, the fact that these experiments could only be carried out in patients with at least 1% of Vδ2 T cells may have represented a selection bias, and our results should be interpreted only in the context of this subset of MDS patients.

We found that PBMC cultures of 60% of the MDS patients responded to BrHPP, with 28% identified as *high responders*, resulting in Vδ2 T-cell expansions similar to those obtained in healthy subjects, while reduced responses to BrHPP were seen in the remaining patients.

In healthy donors, the response to phosphoantigens and cytokines is affected by the differentiation status of γδ T cells: IL-2 stimulates the expansion of T_{CM}, and IL-15 is required for the survival and expansion of T_{EM} cells.[37] In addition, we previously showed poor expansion of Vδ2 T cells following BrHPP+IL-2 stimulation in patients with metastatic renal cell carcinoma with a predominant population of T_{EM}. Similar findings were reported in multiple myeloma patients following stimulation with zoledronic acid+IL-2.[16] In cultures from MDS patients, the response to BrHPP was independent of the γδ T-cell repertoire, and IL-15 did not increase the expansion of effector memory T cells, suggesting that the altered expansion we found in some patients was due to an intrinsic defect of those cells.

In MDS patients responding to BrHPP, the response pattern was nevertheless different from that of healthy donors, and two categories of responders could be distinguished according to the rate of Vδ2 T-cell expansion: *high* responders (generating >70% of Vδ2 T cells, as in controls) and *intermediate* responders (generating only 30-70% of Vδ2 T cells). Expanded Vδ2 T cells from MDS

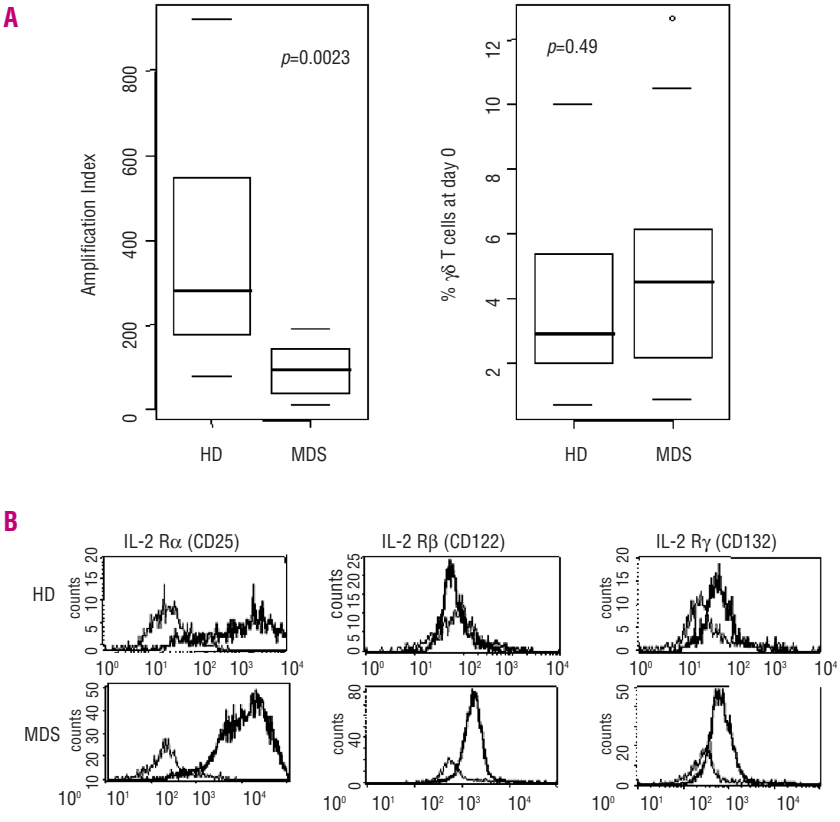

Figure 4. Altered proliferation of Vδ2 T cells despite normal expression and induction of IL-2Rα by MDS patient-derived resting and activated Vδ2 T cells. **A.** Proliferation of Vδ2 T cells in donors (n=11) and MDS patients (n=10) in response to BrHPP and IL-2. Donors and patients with >1% peripheral Vδ2 T cells were studied. The Vδ2 T cells were counted at 3-day intervals during the expansion induced by BrHPP and IL-2, and the proliferation index calculated as described in the *Design and Methods* section. The boxplots on the left show the proliferation index of expanded Vδ2 T cells derived from healthy donors (HD) and MDS patients. The boxplots on the right show percentages of circulating Vδ2 T cell on day 0 from HD and MDS responding patients. **B.** Expression of IL-2Rα, β and γ chains on resting γδ T cells and induction of CD25 IL-2Rα chain on HD and MDS patient-derived Vδ2 T cells treated for 5 days with BrHPP and IL-2. Overlay of resting and activated Vδ2 T cells. One representative experiment of six is shown.

patients expressed lower levels of CD8 than those from controls. In contrast, we previously reported stronger CD8 expression on Vδ2 T cells expanded from renal cell carcinoma patients.[24] These data indicate that different subsets of γδ T cells are expanded in patients with MDS and metastatic renal cell carcinoma. However, activated Vδ2T cells derived from responding MDS patients were functional, efficiently killed target cells including an MDS-derived cell line, and secreted large amounts of interferon-γ. Thus, these activated γδ T cells may act as cytotoxic effectors in the elimination of MDS clones. This is in contrast to MDS-derived NK cells that exhibit altered lytic and secretory capacities, even in response to high doses of IL-2.[9]

In addition, although we previously showed that a significant proportion of peripheral NK cells belonged to the myelodysplastic clone,[9] we detected no MDS cytogenetic abnormalities in γδ T cells expanded *in vitro* from MDS patients. These data are consistent with the ontogeny of T cells, although we cannot exclude the loss of clonal Vδ2 T cells during culture (the low frequency of this peripheral T-cell subset precluded direct *ex vivo* analysis).

Another characteristic of the response to BrHPP in cultures of cells from MDS patients was the low rate of proliferation of the expanded Vδ2 T cells. This change in proliferation *in vitro* in response to IL-2 (and IL-15), similar to that which we previously described for MDS NK cells,[9] was not restored by IL-7 or IL-21, and suggests a defect in the common IL-2/IL-15 signal transduction pathway. We showed that CD25 (IL-2 Rα receptor) was induced by the activation of NK and γδ T cells, suggesting that, despite normal expression of IL-2R, downstream IL-2 signaling pathways may be altered. In human Vδ2T cells, IL-2 activates signal transducer and activator of transcription (STAT) 3 and STAT-5, PI3kinase and ErK2 (as in NK and T-cell receptor α/β T cells), as well as STAT4 (as in NK cells), and p38 mitogen-activated protein kinase (as in T-cell receptor α/β T cells).[38] As proliferation rates of NK and γδ T cells are altered in MDS, we are currently investigating the STAT signaling pathways in these cases.

In conclusion, an expansion of potent activated cytotoxic γδ effectors in response to phosphoantigens could be achieved in 60% of MDS patients, irrespective of cytogenetic findings, WHO subtype or risk category. These expanded cells may be of particular value in the development of immunotherapy for MDS.

Authorship and Disclosures

JJK, GV, EV, SC, VE, JS, JHB, SC, PF and AC all made substantial contributions to the conception and design of the study, acquisition, analysis or interpretation of the data, or drafting the article or revising it critically for important intellectual content; all authors approved the final version to be published.

The authors reported no potential conflicts of interest.

References

1. Heaney M, Golde D. Myelodysplasia. N Engl J Med 1999; 340: 1649-60.
2. Hamblin T. Immunology of the myelodysplastic syndromes. In: Bennet JM, ed. The Myelodysplastic Syndromes: Pathobiology and Clinical Management. New York: Marcel Dekker; 2002. p. 65-7.
3. Hebbar M, Brouillard M, Wattel E, Decoulx M, Hatron PY, Devulder B, et al. Association of myelodysplastic syndrome and relapsing polychondritis: further evidence. Leukemia 1995; 9:731-3.
4. Hebbar M, Kozlowski D, Wattel E, Mastrini S, Dievart M, Duclos B, et al. Association between myelodysplastic syndromes and inflammatory bowel diseases. Report of seven new cases and review of the literature. Leukemia 1997;11:2188-91.
5. Greenberg P, Young N, N Gatterman. Myelodysplastic syndromes. Hematology 2002; 1: 136-61.
6. Molldrem JJ, Leifer E, Bahceci E, Saunthararajah Y, Rivera M, Dunbar C, et al. Antithymocyte globulin for treatment of the bone marrow failure associated with myelodysplastic syndromes. Ann Intern Med 2002; 137:156-63.
7. Albertsson PA, Basse PH, Hokland M, Goldfarb RH, Nagelkerke JF, Nannmark U, et al. NK cells and the tumour microenvironment: implications for NK-cell function and anti-tumour activity. Trends Immunol 2003;24:603-9.
8. Kobayashi H, Tanaka Y, Yagi J, Osaka Y, Nakazawa H, Uchiyama T, et al. Safety profile and anti-tumor effects of adoptive immunotherapy using gamma-delta T cells against advanced renal cell carcinoma: a pilot study. Cancer Immunol Immunother 2007;56:469-76.
9. Kiladjian JJ, Bourgeois E, Lobe I, Braun T, Visentin G, Bourhis JH, et al. Cytolytic function and survival of natural killer cells are severely altered in myelodysplastic syndromes. Leukemia 2006;20:463-70.
10. Band H, Panchamoorthy G, Mclean J, Morita C, Ishikawa S, Modlin R, et al. Recognition of mycobacterial antigens by gamma delta T cells. Res Immunol 1990;141:645-51.
11. Kato Y, Tanaka Y, Miyagawa F, Yamashita S, Minato N. Targeting of tumor cells for human gammadelta T cells by nonpeptide antigens. J Immunol 2001;167:5092-8.
12. Sicard H, Al Saati T, Delsol G, Fournie JJ. Synthetic phosphoantigens enhance human Vgamma9 Vdelta2 T lymphocytes killing of non-Hodgkin's B lymphoma. Mol Med 2001;7:711-22.
13. Gober H, Kistowska M, Angman L, Jeno P, Mori L, Libero GD. Human T cell receptor gammadelta cells recognize endogenous mevalonate metabolites in tumor cells. J Exp Med 2003;197:163-8.
14. Kunzmann V, Bauer E, Feurle J, Weissinger F, Tony HP, Wilhelm M. Stimulation of gammadelta T cells by aminobisphosphonates and induction of antiplasma cell activity in multiple myeloma. Blood 2000; 96:384-92.
15. Kunzmann V, Wilhelm M. Anti-lymphoma effect of gammadelta T cells. Leuk Lymphoma 2005;46:671-80.
16. Mariani S, Muraro M, Pantaleoni F, Fiore F, Nuschak B, Peola S, et al. Effector gammadelta T cells and tumor cells as immune targets of zoledronic acid in multiple myeloma. Leukemia 2005;19:664-70.
17. Das H, Wang L, Kamath A, Bukowski J. Vgamma2Vdelta2 T-cell receptor-mediated recognition of aminobisphosphonates. Blood 2001; 98:1616-8.
18. Brandes M, Willimann K, Moser B. Professional antigen-presentation function by human gammadelta T Cells. Science 2005;309:264-8.
19. Kapp JA, Kapp LM, McKenna KC. Gammadelta T cells play an essential role in several forms of tolerance. Immunol Res 2004;29:93-102.
20. Born WK, Jin N, Aydintug MK,

Wands JM, French JD, Roark CL, et al. gammadelta T lymphocytes-selectable cells within the innate system? J Clin Immunol 2007;27: 133-44.
21. Balbi B, Valle M, Oddera S, Giunti D, Manca F, Rossi G, et al. T-lymphocytes with gamma delta+ V delta 2+ antigen receptors are present in increased proportions in a fraction of patients with tuberculosis or with sarcoidosis. Am Rev Respir Dis 1993;148:1685-90.
22. Janis E, Kaufmann S, Schwartz R, Pardoll D. Activation of gamma delta T cells in the primary immune response to Mycobacterium tuberculosis. Science 1989;244:713-6.
23. Sireci G, Espinosa E, Di Sano C, Dieli F, Fournie JJ, Salerno A. Differential activation of human gammadelta cells by nonpeptide phosphoantigens. Eur J Immunol 2001; 31:1628-1635.
24. Viey E, Fromont G, Escudier B, Morel Y, Da Rocha S, Chouaib S, et al. Phosphostim-activated gamma delta T cells kill autologous metastatic renal cell carcinoma. J Immunol 2005;174:1338-47.
25. Greenberg P, Cox C, LeBeau M, Fenaux P, Morel P, Sanz G, et al. International scoring system for evaluating prognosis in myelodysplastic syndromes. Blood 1997;89: 2079-88.
26. Espinosa E, Belmant C, Pont F, Luciani B, Poupot R, Romagne F, et al. Chemical synthesis and biological activity of bromohydrin pyrophosphate, a potent stimulator of human gamma delta T cells. J Biol Chem 2001;276:18337-44.
27. Dieli F, Poccia F, Lipp M, Sireci G, Caccamo N, Di Sano C, et al. Differentiation of effector/memory Vdelta2 T cells and migratory routes in lymph nodes or inflammatory sites. J Exp Med 2003;198:391-7.
28. Parker CM, Groh V, Band H, Porcelli SA, Morita C, Fabbi M, et al. Evidence for extrathymic changes in the T cell receptor gamma/delta repertoire. J Exp Med 1990;171: 1597-612.
29. Alter G, Malenfant JM, Altfeld M. CD107a as a functional marker for the identification of natural killer cell activity. J Immunol Methods 2004;294:15-22.
30. de Witte T, Oosterveld M, Muus P. Autologous and allogeneic stem cell transplantation for myelodysplastic syndrome. Blood Rev 2007;21:49-59.
31. Maciejewski JP, Anderson S, Katevas P, Young NS. Phenotypic and functional analysis of bone marrow progenitor cell compartment in bone marrow failure. Br J Haematol 1994; 87:227-34.
32. Marisavljevic D, Kraguljac N, Rolovic Z. Immunologic abnormalities in myelodysplastic syndromes: clinical features and characteristics of the lymphoid population. Med Oncol 2006;23: 385-92.
33. Giacomelli R, Cipriani P, Fulminis A, Barattelli G, Matucci-Cerinic M, D'Alo S, et al. Circulating g/d T lymphocytes from systemic sclerosis (SSc) patients display a T helper (Th) 1 polarization. Clin Exp Immunol 2001;125: 310-5.
34. Alaibac M, Berti E, Chizzolini C, Fineschi S, Marzano AV, Pigozzi B, et al. Role of cellular immunity in the pathogenesis of autoimmune skin diseases. Clin Exp Rheumatol 2006;24:S14-9.
35. Riccieri V, Spadaro A, Parisi G, Taccari E, Moretti T, Bernardini G, et al. Down-regulation of natural killer cells and of gamma/delta T cells in systemic lupus erythematosus. Does it correlate to autoimmunity and to laboratory indices of disease activity? Lupus 2000;9:333-7.
36. Re F, Poccia F, Donnini A, Bartozzi B, Bernardini G, Provinciali M. Skewed representation of functionally distinct populations of Vg9Vd2 T lymphocytes in aging. Exp Gerontol 2005;40:59-66.
37. Caccamo N, Meraviglia S, Ferlazzo V, Angelini D, Borsellino G, Poccia F, et al. Differential requirements for antigen or homeostatic cytokines for proliferation and differentiation of human Vg9Vd2 naive, memory and effector T cell subsets. Eur J Immunol 2005;35:1764-72.
38. Lafont V, Loisel S, Liautard J, Dudal S, Sable-Teychene M, Liautard JP, et al. Specific signaling pathways triggered by IL-2 in human Vg9Va2 T cells: an amalgamation of NK and ab T cell signaling. J Immunol 2003; 171:5225-32.