

Phase I clinical trial combining imatinib mesylate and IL-2

HLA-DR⁺ NK cell levels correlate with disease outcome

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Abbreviations: AUC, area under the curve; C_{max} , maximal concentration; CTX, cyclophosphamide; GIST, gastrointestinal stromal tumor; IM, imatinib mesylate; IL, interleukin; NK, natural killer; PBMC, peripheral blood mononuclear cell; PFS, progression-free survival; OS, overall survival; TRAIL, tumor necrosis factor-related apoptosis-inducing ligand; Treg, regulatory T cell

We performed a Phase I clinical trial from October 2007 to October 2009, enrolling patients affected by refractory solid tumors, to determine the maximum tolerated dose (MTD) of interleukin (IL)-2 combined with low dose cyclophosphamide (CTX) and imatinib mesylate (IM). In a companion paper published in this issue of *Oncolmunology*, we show that the MTD of IL-2 is 6 MIU/day for 5 consecutive days, and that IL-2 increases the impregnation of both IM and of its main metabolite, CGP74588. Among the secondary objectives, we wanted to determine immunological markers that might be associated with progression-free survival (PFS) and/or overall survival (OS). The combination therapy markedly reduced the absolute counts of B, CD4⁺ T and CD8⁺ T cells in a manner that was proportional to IL-2 dose. There was a slight (less than 2-fold) increase in the proportion of regulatory T cells (Tregs) among CD4⁺ T cells in response to IM plus IL-2. The natural killer (NK)-cell compartment was activated, exhibiting a significant upregulation of HLA-DR, tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) and CD56. The abundance of HLA-DR⁺ NK cells after one course of combination therapy positively correlated with both PFS and OS. The IL-2-induced rise of the CD4⁺:CD8⁺ T-cell ratio calculated after the first cycle of treatment was also positively associated with OS. Overall, the combination of IM and IL-2 promoted the rapid expansion of HLA-DR⁺ NK cells and increased the CD4⁺:CD8⁺ T-cell ratio, both being associated with clinical benefits. This combinatorial regimen warrants further investigation in Phase II clinical trials, possibly in patients affected by gastrointestinal stromal tumors, a setting in which T and NK cells may play an important therapeutic role.

Introduction

Although cancer immunotherapy has been used for a number of years, new strategies, including the blockage of immune checkpoints, have recently emerged. Combinational regimens involving several chemotherapeutic constitute a mainstay of oncology. Now, combining immunotherapeutic approaches with

conventional chemotherapy stands out as promising strategies to increase response rates, circumvent chemoresistance and hence prolong patient survival. The identification of novel combination regimens and their characterization in terms of optimal dosage and schedule should help achieving these goals. Beyond the intrinsic capacity of a targeted anticancer agent to interfere with oncogene addiction and hence promote apoptosis, often

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unrecognized off-target activities of the drug under consideration on the key players of innate and cognate immunity dramatically influence therapeutic responses. Thus, the association of immunogenic chemotherapy with antibodies blocking major immune checkpoints, immunomodulators (such as Toll-like receptor agonists or cytokines), therapeutic vaccines, or adoptive T-cell transfer constitutes a promising immunochemotherapeutic approach.

Based on previous preclinical studies,¹⁻⁴ we developed an innovative strategy aimed at activating both T and natural killer (NK) cells, which combines metronomic cyclophosphamide (CTX), imatinib mesylate (IM) and interleukin (IL)-2. CTX is a DNA-alkylating agent belonging to the family of nitrogen mustards. Upon conversion to 4-hydroxycyclophosphamide by hepatic oxidases, CTX acquires cytotoxic properties.⁵ Recent data indicate that, in contrast to the immunosuppressive properties of CTX at high doses, metronomic CTX regimens exert profound immunostimulatory and anti-angiogenic effects.^{1,6,7} CTX controls dendritic cell (DC) homeostasis, promotes the secretion of interferon (IFN) α ,⁷ contributes to the induction of antitumor cytotoxic T lymphocytes (CTLs)⁸ and to the proliferation of adoptively transferred T cells, induces the polarization of CD4⁺ T cells toward T_H1 and/or T_H17 lymphocytes,⁹ and reduced the abundance and functions of regulatory T cells (Treg), eventually favoring tumor regression.¹ Such immunostimulatory properties offer a new approach for the use of metronomic CTX, as current clinical successes obtained with CTX are based on high doses, which de facto are immunosuppressive.

The tyrosine kinase inhibitor IM was developed in the late 1990s as a targeted therapy for chronic myeloid leukemia patients bearing the *BCR-ABL* genetic rearrangement.¹⁰⁻¹³ IM was first administered to patients with gastrointestinal stromal tumors (GIST) in 2001,¹⁴ and—rapidly—two international Phase III clinical trials showed that IM can achieve disease control in 70–85% of patients with advanced GIST bearing *KIT* or *PDGFRA* (coding for the platelet-derived growth factor receptor α subunit) mutations, with a median progression-free survival (PFS) of 20–24 mo.¹⁵⁻¹⁷ A number of immunological off-target effects have been reported for IM. IM can affect Treg functions by inhibiting the expression of forkhead box P3 (FOXP3), thereby enhancing the immunogenicity of anticancer vaccines.¹⁸ By inhibiting oncogenic KIT signaling, IM was shown to shut down the expression of the immunosuppressive enzyme indoleamine 2,3-dioxygenase (IDO) by tumor cells, thereby promoting Treg apoptosis and CD8⁺ T-cell activation in human and murine GIST models.¹⁹ We have previously reported that IM promote the DC/NK-cell cross-talk by acting on KIT-expressing bone marrow DCs, hence endowing them with the capacity to stimulate NK cells.²⁰ In animals bearing IM-resistant tumors, oral IM has been shown to promote the proliferation and activation of NK cells in the spleen concomitant to an NK cell-dependent reduction of the metastatic burden.²⁰ In GIST patients treated with IM for 2 mo, the DC-induced secretion of IFN γ by NK cells turned out to constitute a predictor of long-term therapeutic responses.^{20,21} Finally, combining IM with IL-2 in mice bearing IM-resistant metastatic

melanoma produced a synergistic anticancer effect.³ In particular, high doses of IL-2 boosted the antitumor effects mediated by IM, via a mechanism dependent on NK1.1⁺ cells, tumor necrosis factor-related apoptosis-inducing ligand (TRAIL), and IFN γ . Such antitumor effects were associated with the intratumoral recruitment of a particular subset of innate immune cells harboring a hybrid phenotype between DCs and NK cells (MHC Class II⁺CD11c⁺NK1.1⁺B220⁺), which we named ‘interferon-producing killer dendritic cells’ (IKDCs). Indeed, IKDCs were capable of secreting IFN γ in response to tumor cells upon stimulation with IM and IL-2, as well as of killing tumor cell targets by a TRAIL-dependent mechanism.³ The antitumor efficacy of IM plus IL-2 was compromised in mice bearing loss-of-function mutations in the IL-15 receptor α (IL-15R α) or in Type I IFNs receptor 1, and was dependent on plasmacytoid DCs. IL-15R α was required for the proliferation of IKDCs in the course of therapy with IM plus IL-2. The trans-presentation of IL-15 induced the expression of the C-C chemokine receptor Type 2 (CCR2) on IKDCs, and primed IKDCs to respond to Type I IFN by producing chemokine (C-C) ligand 2 (CCL2). Of note, the antitumor effects of IM plus IL-2 correlated with a CCL2-dependent recruitment of IKDCs but not B220⁺ NK cells into the tumor bed.^{2,22} We concluded that the IL-15-driven peripheral expansion and the CCL2-dependent intratumoral targeting of IKDCs constitute the checkpoints that dictate the antitumor efficacy of IM plus IL-2 in mice.

Given that Tregs inhibit NK-cell effector functions,²³ and that CTX impairs Treg homeostasis, restoring NK-cell functions,¹ we have conducted a Phase I trial combining CTX, IM and escalating doses of IL-2 (see companion paper published in *OncolImmunology* 2:e23079). One of the secondary objectives of this trial was to identify a human analog of mouse IKDCs or alternative immune parameters associated with clinical benefits in patients affected by refractory solid malignancies.

Results

The combination of IM and IL-2 drastically reduces the counts of most lymphocyte subsets. Most patients presented with lymphopenia at enrollment ($1118 \pm 100/\text{mm}^3$), which worsened significantly ($p < 0.001$) after one cycle of therapy ($600 \pm 70/\text{mm}^3$). This lymphopenic effect was attributable to the addition of IL-2, as no worsening was seen after CTX or IM (Fig. 1A; Table S1). The abundance of lymphocyte subsets including CD4⁺ T cells, CD8⁺ T cells, and B lymphocytes dropped (Fig. 1B–D; Table S1), whereas the absolute counts of NK and Treg cells remained stable (Fig. 2A and C; Table S1), resulting in an increased proportion of Treg and NK cells following the administration of IM plus IL-2 (Fig. 2B and D; Table S1). At the end of the first cycle of treatment (D14), there was an inverse correlation between the dose of IL-2/kg and the total number of lymphocytes ($R = -0.6432$; $p = 0.009$), CD8⁺ T cells ($R = -0.6899$; $p = 0.004$) and NK cells ($R = -0.6470$; $p = 0.009$), as well as with Treg counts/ mm^3 ($R = -0.5255$; $p = 0.04$) (Fig. 1F and 2F). Interestingly, and as already reported in HIV individuals receiving IL-2,²⁴ the CD4⁺:CD8⁺ T-cell ratio was increased at the end

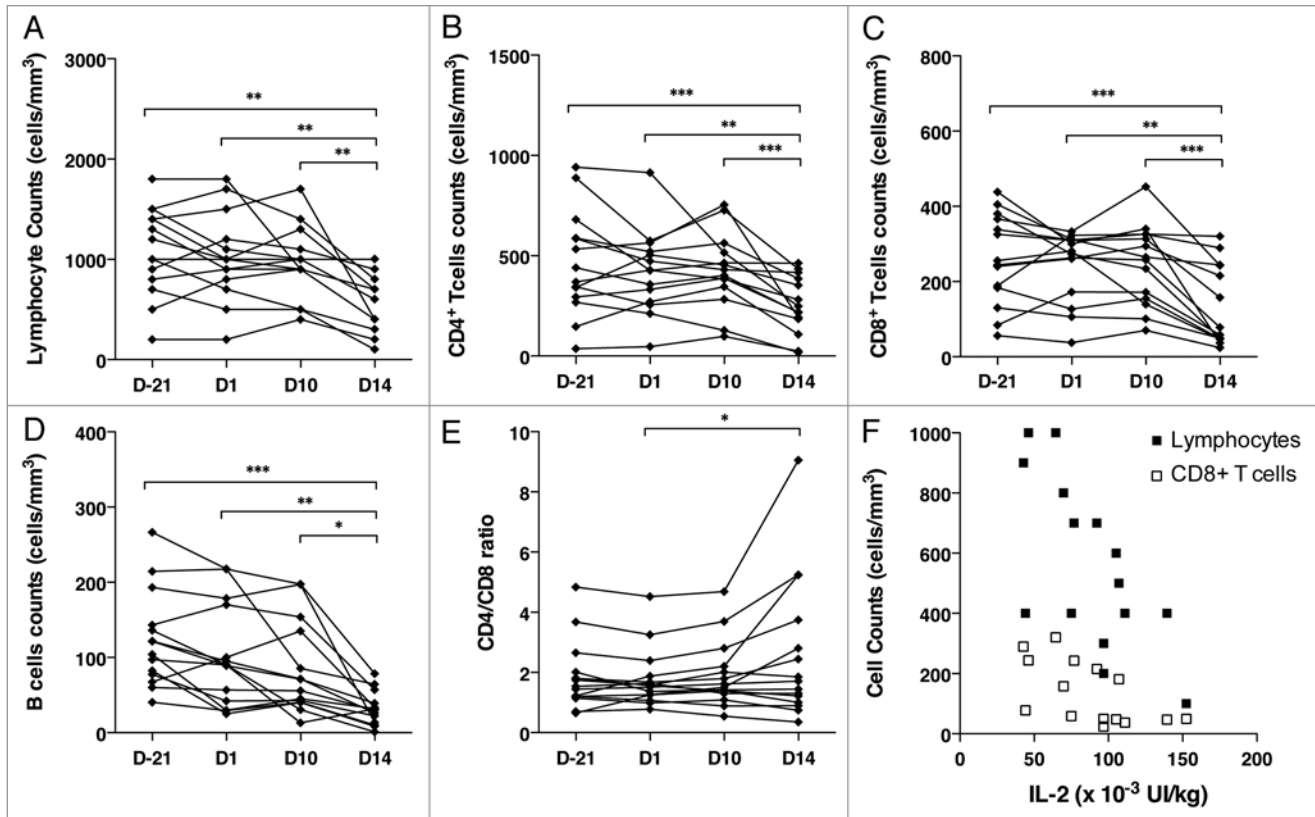


Figure 1. The drop in lymphocyte counts observed during imatinib mesylate + interleukin-2 (IL-2) therapy correlated with IL-2 dose. (A–F) Cytofluorometric quantification of all lymphocytes (A), CD3⁺CD4⁺ T cells (B), CD3⁺CD8⁺ T cells (C) and CD3⁺CD19⁺ B cells (D) in the blood of patients at baseline (D-21), before the initiation of imatinib mesylate (IM, D1), before the initiation of interleukin-2 (IL-2, D10) and at the completion of the first treatment cycle (D14). The CD4⁺:CD8⁺ T-cell ratio was determined at each point (E). At D14, lymphocyte and CD8⁺ cell counts inversely correlated with IL-2 dose (F). Each dot represents the parameters of one patient. Statistical significance is indicated (**p* < 0.05, ***p* < 0.01, ****p* < 0.001).

of the combination therapy (*p* = 0.015; Fig. 1E). The relative abundance of Tregs decreased slightly after the administration of CTX and IM (*p* = 0.04; Fig. 2D). Hence, despite the use of IL-2,^{25,26} we observed an increase greater than 2-fold in the proportion of Tregs (among CD4⁺ T cells) in three patients (representing 20% of this cohort) after one cycle of therapy (Fig. 2F).

The combination of IM and IL-2 activates circulating NK cells. The activation of NK cells was assessed not only based on their expansion among all lymphocytes (Fig. 2A and C; Table S1) but also on the overexpression of CD56 (Fig. 3A; Table S1), HLA-DR (Fig. 3B and Table S1) and TRAIL (Fig. 3C; Table S1). Of note, HLA-DR molecules were mainly upregulated by the CD56^{bright} NK-cell subset (Fig. 3D). The upregulation of all these activation markers was attributable to IL-2 (Fig. 3A–C), but the increased abundance of CD56^{bright} NK cells was already significant at D10, when patients had not yet received IL-2 (Fig. 3A). There was a positive correlation between the IL-2 dose/kg and the expression of TRAIL by NK cells (*R* = 0.68; *p* = 0.004, Fig. 3E). Corroborating the results of cytofluorometric studies, the cytolytic functions of NK cells, as assessed by CD107a expression upon exposure to K562 cells, increased in response to the combinatorial regimen (Fig. 3F), whereas IFN γ secretion remained stable (data not shown).

Early parameters associated with clinical outcome. We next evaluated which among the T cell-related and NK cell-related immunological parameters monitored at each time point during the first cycle of treatment (D-21, D1, D10 and D14) would be associated with PFS and/or OS. For each parameter, we defined the median as the cut-off value at each time point.

The ratio of CD4⁺:CD8⁺ T cells observed after the CTX treatment (D1) predicted OS (*p* = 0.0321), and this trend was reinforced after IM (D10, *p* = 0.0015) and sustained after IL-2 (D14, *p* = 0.0015) (Fig. 4A–D). However, CD4⁺:CD8⁺ T-cell ratio failed to predict PFS in this clinical cohort. Neither the proportion of NK cells (defined as CD56⁺CD3⁻ lymphocytes) nor CD56 (proportion of CD56^{dim} NK cells or CD56^{bright} NK cells among CD56⁺CD3⁻ NK cells) or TRAIL expression on NK cells had a predictive value for PFS or OS (not shown). By contrast, the proportion of HLA-DR⁺ NK cells observed after IM therapy (D10) was positively associated with PFS (*p* = 0.008; Fig. 5A) and OS (*p* = 0.01; Fig. 5B). This trend was reinforced at D14 for OS (*p* = 0.009; Fig. 5C). Notably, among HLA-DR⁺ NK cells, only a high proportion of CD56^{bright}HLA-DR⁺ (but not of CD56^{dim}HLA-DR⁺) NK cells remained associated with prolonged OS after IM plus IL-2 therapy (D14, *p* = 0.009; Fig. 5D). IL-2-specific evaluation criteria such as the abundance of Tregs

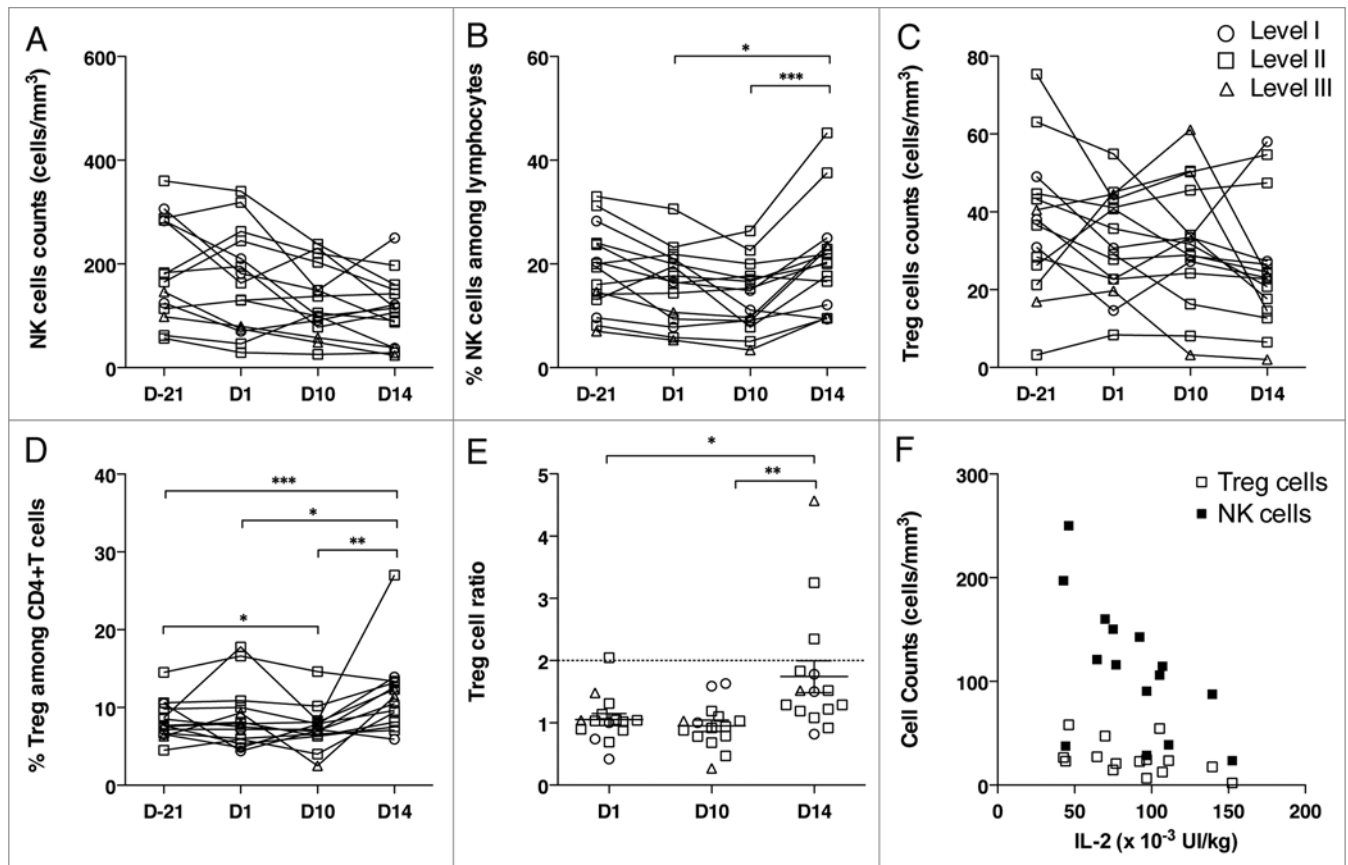


Figure 2. Interleukin-2-induced a rise in the proportions of natural killer and regulatory T cells. (A–F) Cytofluorometric quantification of natural killer (NK, CD3⁺CD56⁺) cells among all lymphocytes (A and C) and regulatory T cells (Tregs, CD3⁺CD4⁺CD127^{low}CD25^{high}) among CD3⁺CD4⁺ T cells (B and D) in the blood of patients at baseline (D-21), before the initiation of imatinib mesylate (IM, D1), before the initiation of interleukin-2 (IL-2, D10) and at the completion of the first treatment cycle (D14). The fold increase in Treg (determined by the ratio of Treg relative abundance between subsequent time points (D1/D-21; D10/D1; D14/D10)) is depicted (E). NK cell and Treg levels numbers inversely correlated with IL-2 dose (F). Each dot represents the parameters of one patient. Statistical significance is indicated (**p* < 0.05, ***p* < 0.01, ****p* < 0.001).

(Fig. 2) or the circulating levels of soluble CD25 (see companion paper published in *OncImmunology* 2:e23079) failed to correlate with PFS or OS (data not shown).

Discussion

The rationale for combining IM and IL-2 in this study relied upon the NK-cell immunostimulatory capacities of IM and upon preclinical studies demonstrating that IKDCs may mediate the TRAIL- and IFN γ -dependent tumoricidal activity of IM when combined with high doses of IL-2.³ The results of this Phase I clinical trial indicate that (1) IL-2 can only be used at an intermediate dose (i.e., 6 MIU/day) when combined with IM (see companion paper published in *OncImmunology* 2:e23079); (2) the combination of IM and IL-2 triggers NK-cell expansion and activation; (3) the ratio of CD4⁺:CD8⁺ T cells after CTX treatment may predict patient OS; (4) Tregs are not markedly by the combination of IM and IL-2, in spite of the presence of the latter, nor are they associated with disease outcome and (5) the presence of HLA-DR⁺ and particularly CD56^{bright} HLA-DR⁺ NK cells after IM and IM plus IL-2 therapy is associated with PFS and/or OS.

Low doses of rIL-2 can activate high-affinity IL-2R α ⁺ CD56^{bright} NK cells, favor NK-cell differentiation from IL-2R α ⁺ CD34⁺ NK-cell precursors, and increase the proportions and functions of CD4⁺CD25⁺ regulatory T cells. Therefore, the question arises as to whether IM might have a positive effect on NK-cell function and/or a negative effect on Treg abundance or function, and hence bring about clinical benefits for cancer patients. It has previously been demonstrated that IM exerts inhibitory effects on the immunosuppressive activity of CD4⁺CD25^{high} T cells. Balachandran et al. reported that—in a GIST mouse tumor model and in GIST patients bearing *KIT* mutations—IM hampers IDO activity, thereby promoting the apoptotic demise of tumor-infiltrating Tregs and increasing the CD8⁺ T-cell:Treg ratio in the tumor bed.¹⁹ These authors attributed the therapeutic success of IM at least partly to the reinvigoration of CD8⁺ T cells in the absence of Tregs, an effect that could be boosted by blocking cytotoxic T lymphocyte antigen 4 (CTLA). Larmonier and collaborators demonstrated that IM directly affects FOXP3 expression, signal transducer and activator of transcription (STAT)3 and STAT5 activation, as well as the phosphorylation of ZAP70 (ζ chain-associated protein kinase 70) and LAT (linker

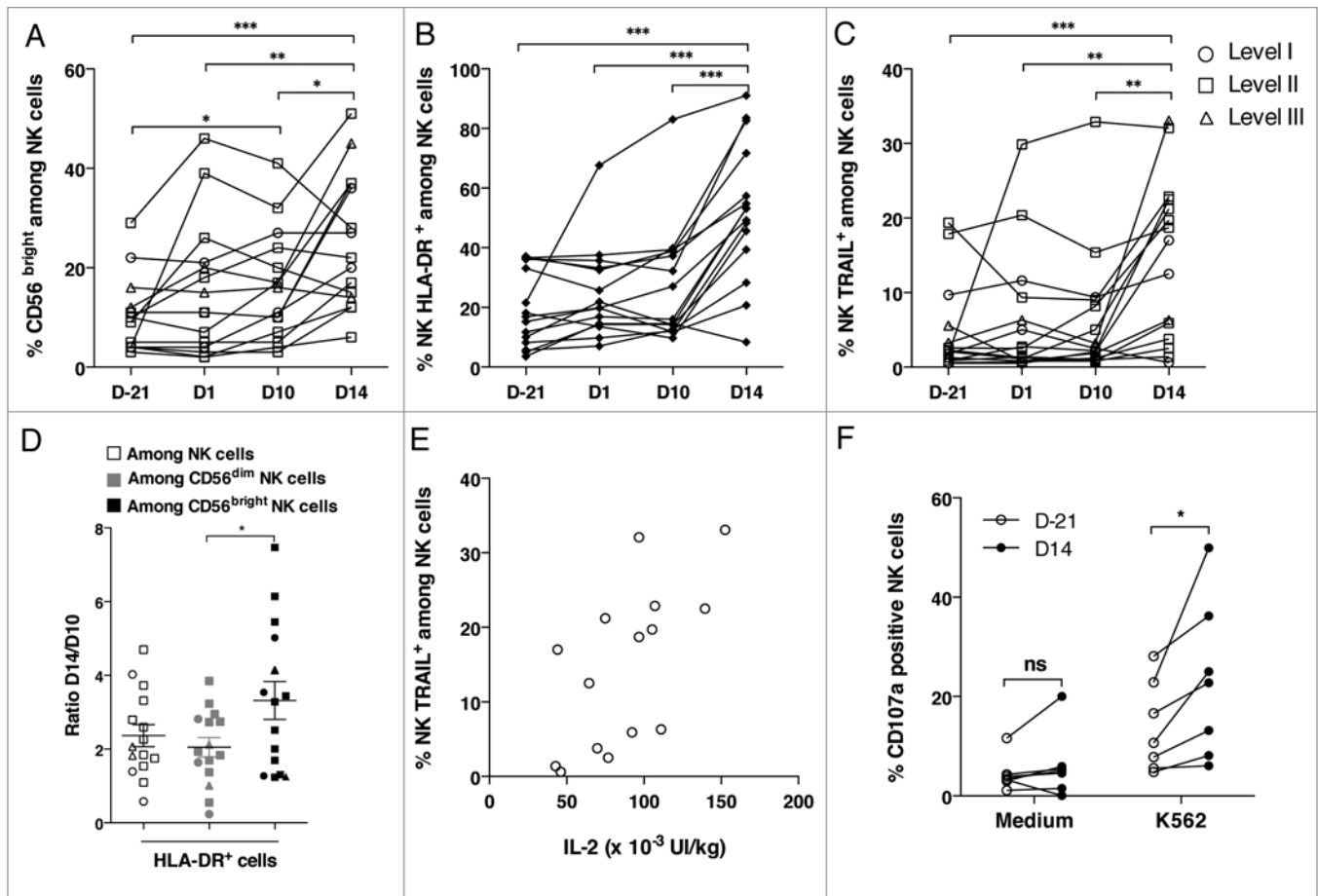


Figure 3. Activation of peripheral natural killer cells in response to imatinib mesylate alone or combined with interleukin-2. (A–F) Cytofluorometric quantification of CD56 (A), HLA-DR (B), and TRAIL (C) expression on CD3⁺CD56⁺ lymphocytes in the blood of patients at baseline (D-21), before the initiation of imatinib mesylate (IM, D1), before the initiation of interleukin-2 (IL-2, D10) and at the completion of the first treatment cycle (D14). Fold increase from D10 to D14 (D14/D10) of the proportion of HLA-DR⁺ cells among CD3⁺CD56⁺, CD3⁺CD56^{dim} and CD3⁺CD56^{bright} natural killer (NK) cells (D). The percentage of TRAIL-expressing NK cells positively correlated with IL-2 dose (E). (F) Degranulation capacity of NK cells (assessed by CD107a expression) obtained from patients at baseline (D-21) and after one cycle of treatment (D14), upon exposure to K562 cells. Each dot represents the parameters of one patient. Statistical significance is indicated (**p* < 0.05, ***p* < 0.01, ****p* < 0.001).

for activation of T cells) in human Tregs in vitro, at clinically relevant concentrations, and can impair the frequency and function of Tregs in mice.¹⁸ In the current study, we found that: (i) CTX and IM treatment can reduce the proportion of regulatory T cells in patients with advanced cancer (Fig. 2D); and (ii) IM plus 6 MIU/day IL-2 (our MTD) increased the relative abundance of Tregs only slightly, with a change of less than 2-fold on average: 1.5 ± 0.68 (0.82–4.57) (Fig. 2E). These data contrast with those previously reported for patients affected by autoimmune or chronic inflammatory conditions. Saadoun et al. described the use of low doses of IL-2 (1.5 MIU/day for 5 consecutive days in the first cycle, followed by 3 MIU/day for 5 d in cycles 2–4) for the treatment of hepatitis C virus (HCV)-induced vasculitis, and observed a 2-fold increase (after the first cycle) and a 3–4-fold increase (after the second, third and fourth cycle) in Tregs.²⁶ Koreth et al. reported that—in patients affected by grafts-vs.-host disease—low doses of IL-2 (1 MIU/m²/day for 8 weeks) can expand Tregs by eight times, hence causing a relief of symptoms in 12 out of 23 patients.²⁵ The three patients in our cohort

who received the lowest dose of IL-2 (3 MIU/day, i.e., 1.7–2 MIU/m²/day) had an increase in Treg abundance of only 0.82, 1.5, and 1.78 times, respectively. We ascribe such differences to (1) the adjunctive therapy with IM, which might mediate pharmacodynamic changes in IL-2 bioactivity or directly reduce Treg numbers; (2) differences in the schedule of IL-2 administration; (3) the CTX-based conditioning regimen (which is known to interfere with Treg number and function) and (4) patient characteristics (cancer patients have comparatively higher Treg numbers at baseline). We were able to exclude the possibility that IM interfered with the pharmacokinetics or pharmacodynamics of IL-2 in our Phase I study by demonstrating that the peak concentrations and half-life of IL-2 as observed in patients co-treated with IM were similar to those documented for the subcutaneous injection of IL-2 only (see companion paper published in *OncolImmunology* 2:e23079), and that the circulating concentrations of soluble CD25 were in the same range as those observed by Saadoun et al. (D. Klatzmann, personal communication). However, in contrast with our previous study,²⁷ a Phase I clinical

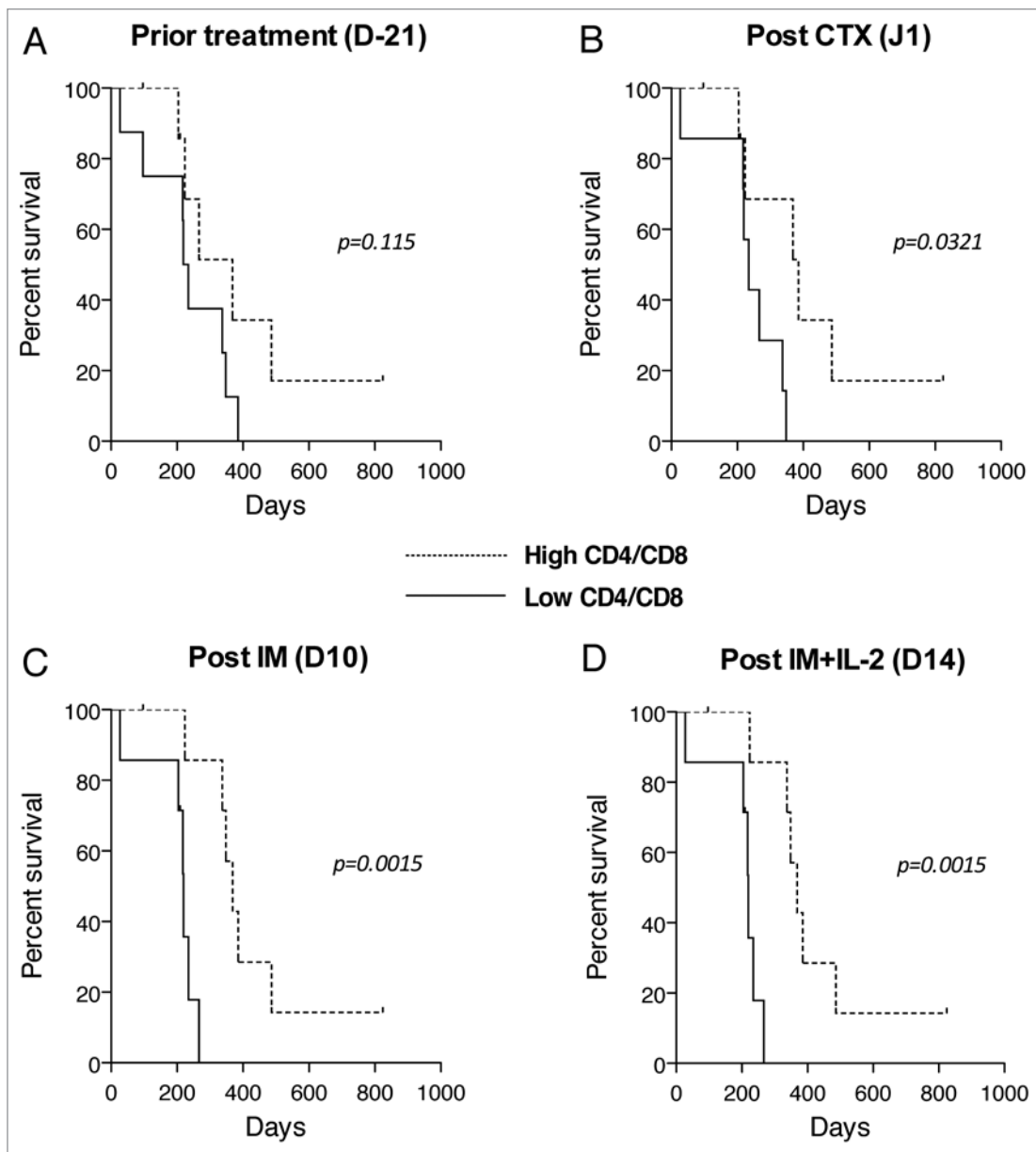


Figure 4. The CD4⁺:CD8⁺ T-cell ratio is a predictor of overall survival post-cyclophosphamide. (A–D) The median of the CD4⁺:CD8⁺ T-cell ratio measured at each time point, that is baseline (D-21), before the initiation of imatinib mesylate (IM, D1), before the initiation of interleukin-2 (IL-2, D10) and at the completion of the first treatment cycle (D14), was used as a cut-off value to determine two groups of patients, high CD4⁺:CD8⁺ patients (value > median, dotted line) and low CD4⁺:CD8⁺ patients (value < median, continuous line). Overall survival in these two groups was compared by means of Mantel-Cox log-rank tests. Curves and p values are shown for D-21 (A), D1 (B), D10 (C) and D14 (D).

trial combining stem cell factor (SCF, a KIT ligand) with IL-2 in HIV-1-infected and cancer patients reported an expansion of NK cells (2.2-fold, for the CD56^{bright} subset) but a 6-fold rise in Tregs. Hence, it remains speculative whether, in the course of IL-2 therapy, blocking c-KIT signaling would favor the expansion of NK cells over that of Tregs while the administration of SCF plus IL-2 would cause Tregs to preferentially expand.²⁸

Our study highlighted the predictive role of the CD4⁺:CD8⁺ T-cell ratio on OS. Indeed, one of the hallmarks of IL-2 bioactivity in HIV-1-infected patients receiving low IL-2 doses was the reconstitution of the CD4⁺ T cell compartment, possibly by

neo-thymopoiesis.²⁴ To address this question, Levy et al. comprehensively investigated the nature of IL-2-induced CD4⁺ T cells, and showed that the administration of IL-2 prior to the interruption of highly active antiretroviral therapy (HAART) during non-advanced HIV disease prolongs the period before HAART resumption as well as survival, in spite of the presence of replicating HIV-1. These effects of IL-2 may stem from the expansion of CD4⁺CD45RO⁻CD25⁺FOXP3⁺ cells exerting weak immunosuppressive functions, as well as that of naive and central memory CD4⁺ T cells.²⁹⁻³¹ In another study, HAART alone was compared with HAART plus IL-2 (6 MIU/day on days 1–5 and 8–12 of a 28

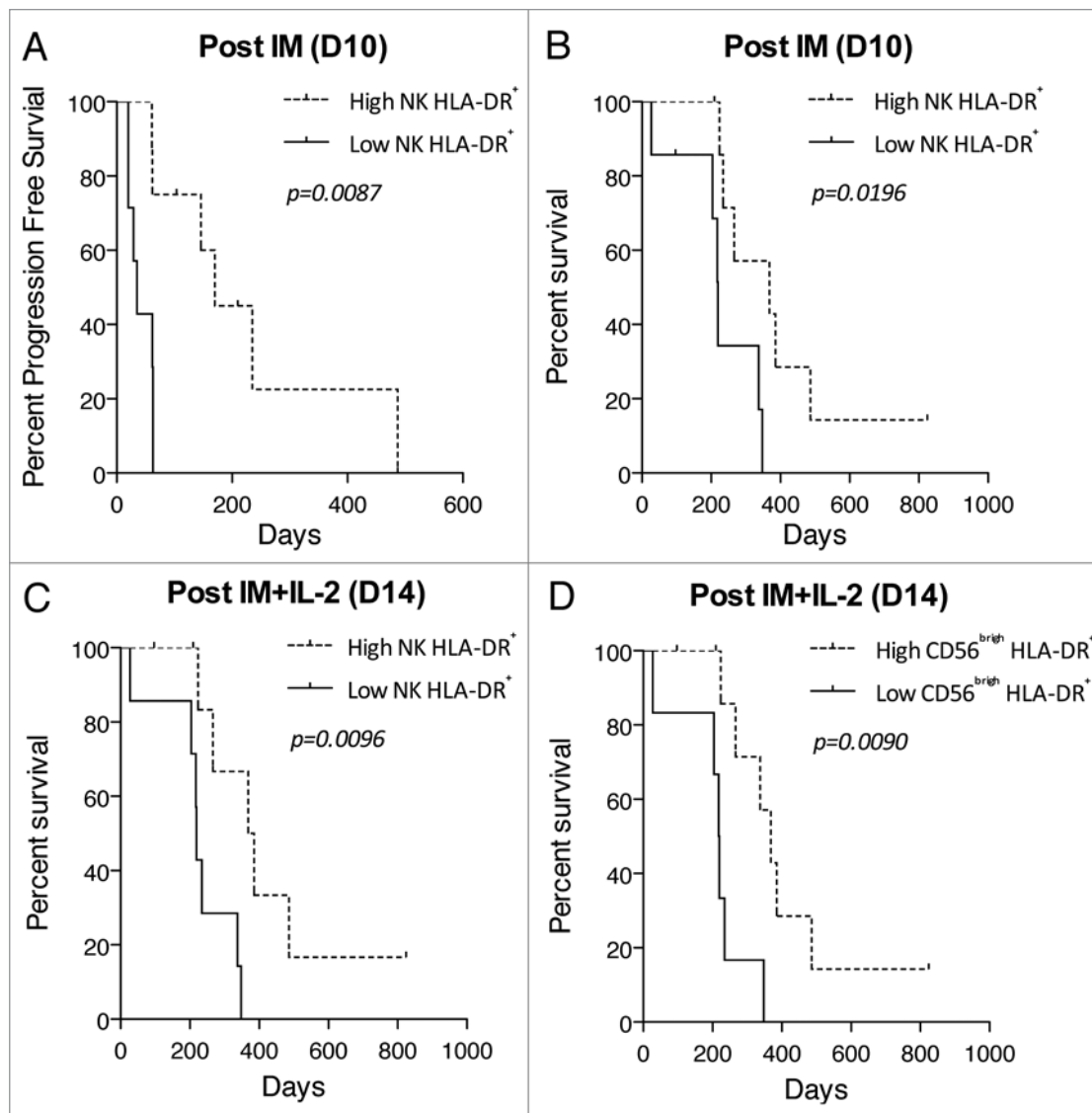


Figure 5. HLA-DR⁺ natural killer cell levels are positively associated with progression-free and overall survival. (A–D) The median of the relative abundance of HLA-DR⁺ or CD56^{bright} HLA-DR⁺ natural killer (NK) measured at each time point, that is baseline (D-21), before the initiation of imatinib mesylate (IM, D1), before the initiation of interleukin-2 (IL-2, D10) and at the completion of the first treatment cycle (D14), was used as a cut-off value to determine two groups of patients, high NK HLA-DR⁺ or high CD56^{bright} HLA-DR⁺ patients (values > median, dotted lines) and low NK HLA-DR⁺ or low CD56^{bright} HLA-DR⁺ patients (values < median, continuous lines). Progression-free survival (PFS) and overall survival (OS) in these two groups were compared by means of Mantel-Cox log-rank tests. Curves and p values depicting PFS are shown for HLA-DR⁺ NK cells at D10 (A). Curves and p values depicting OS are shown for HLA-DR⁺ NK cells at D10 (B) and D14 (C), as well as for CD56^{bright} HLA-DR⁺ NK cells at D14 (D).

d-cycle for a total of six cycles).³² In these HIV-1-infected patients, the authors observed a marked induction of CD4⁺ T cells, leading to an increased CD4⁺:CD8⁺ T-cell ratio, a drop in viremia after 24 weeks, and a 3–4-fold increase in CD4⁺CD25⁺ T cells.³² In our study, after one cycle of treatment, we could not detect any increase in the number or frequency of CD4⁺ or CD8⁺ T cells (Fig. 1). An increase in CD4⁺:CD8⁺ T-cell ratio was observed only after the administration of IM plus IL-2, but a higher ratio after CTX treatment appeared to be linked to OS, and was maintained throughout treatment (post-IM and post-IM plus IL-2; Fig. 4). This observation might stem from the development of T_H1/T_H17-skewed TCR-dependent responses in response to CTX,

as previously described for this cohort of patients,⁹ which could be maintained and/or increased by IM or IM plus IL-2. However, this hypothesis remains to be formally addressed.

The results of this clinical trial indicate that NK-cell-related biomarkers (CD56, HLA-DR and TRAIL) are significantly modulated by the combination of IM plus IL-2. Caligiuri et al. pioneered this field, showing that low doses of IL-2 induce the accumulation of CD56⁺ NK cells, while periodic intermediate-dose pulsing can boost their cytotoxic functions. The degree of IL-2-induced NK-cell expansion was somewhat correlated with the clinical benefit of cancer patients, and was negatively associated with the development of neutralizing anti-IL-2

antibodies.³³⁻³⁶ Similar findings were obtained in HIV-1-infected patients,³⁷ who manifested a dramatic increase in CD56^{bright} NK cells.³⁶ How does IM affect the expansion or activation of NK cells as induced by IM? Although the absolute counts of circulating NK cells failed to increase, the proportion of NK cells among lymphocytes as well as the abundance of CD56^{bright} or HLA-DR⁺ or TRAIL⁺ NK cells among total NK cells raised by 2-fold, 2–3-fold, 2–3-fold and 4-fold, respectively. Therefore, it is tempting to speculate that uncoupling the innate immunostimulatory effects of IL-2 from its Treg-enhancing effects can be achieved by combining it with IM. Chen et al. combined IM with high doses of pegylated (peg)IFN α 2b for 4 weeks in patients with Stage III/IV GIST and reported on the first eight patients who exhibited an objective response. PegIFN α 2b promoted a T_H1 polarization of circulating T and NK cells and tumor infiltration by granzyme B- and FASL-expressing CD56⁺ cells, colocalizing with CD45RO⁺CD8⁺ or CD4⁺ cells.³⁸ It remains unclear how IL-2 vs. pegIFN α 2b differentially activate T and NK cells in GIST patients undergoing IM-based therapy. In our study, IM combined with IL-2 appeared to preferentially amplify CD56^{bright} HLA-DR⁺ NK cells. As reported by others, HLA-DR could mark a subset of human NK cells that do not simply undergo activation, contrary to CD69, which is upregulated on the vast majority of NK cells within 18 h of IL-2 stimulation.^{39,40} Because NK cells derived by clonal expansion of a single seed cell are relatively homogeneous in the expression levels of HLA-DR, Evans and colleagues have suggested that HLA-DR⁺ NK cells that are expanded by IL-2 *ex vivo* may originate from a HLA-DR-expressing precursor cell.³⁹ These HLA-DR⁺ NK cells are more potent than their HLA-DR⁻ counterparts with regard to proliferation and degranulation (but not IFN γ secretion) in response to IL-2 or IL-15.³⁹ In line with these data, we found that HLA-DR⁺ NK cells as induced by IM plus IL-2 therapy exhibit a greater capacity for degranulation but no enhanced IFN γ secretion. Human HLA-DR⁺ NK cells could be analogous to murine IKDCs.³ To date, several groups have shown that NK cells expressing MHC Class II molecules might represent a distinct subset of immature NK cells in mice and humans.^{39,41,42} In the current study, we have demonstrated that the combination of IM plus IL-2 in patients with advanced cancer induces the expansion of HLA-DR⁺TRAIL⁺ NK cells exhibiting potent degranulation capacity, yet only the expansion of HLA-DR⁺ NK cells was positively associated with disease outcome. The study of the transcriptional profile, migratory pattern and antigen-presenting functions of HLA-DR⁺TRAIL⁺ NK cells should unravel whether these cells, as expanded by IM plus IL-2 therapy, truly represent the human analog of IKDCs that we and others described in 2006.^{3,43}

The combination of CTX, IM and IL-2 constitutes a T- and NK-cell immunomodulatory regimen suitable for patients bearing advanced solid malignancies. We found that T and NK cell-related parameters can be linked to PFS and/or OS in these patients. There appears to be no link of causality between these biomarkers of OS, since no measurements were done beyond day 14, implying that we could not demonstrate that these biomarkers are maintained at subsequent cycles. Still, we believe that

this immunotherapeutic regimen could be of interest in patients affected by GIST or chronic myeloid leukemia, two settings in which it could synergize with oncogene addiction-targeting agents.

Materials and Methods

Patients. Adult patients with measurable or evaluable solid malignancies that were refractory to standard therapy were eligible for this study, a Phase I clinical trial (IMAIL-2) run at the Gustave-Roussy Institute and approved by the Kremlin Bicêtre Hospital ethics committee (number 07-019) as well as by the Agence Française de Sécurité Sanitaire des Produits de Santé (A70385-27) in 2007. The patients enrolled in the IMAIL-2 trial and the treatment schedule have been exhaustively described in the companion paper (*Oncol Immunology* 2:e23079).

Blood sampling. Blood was collected at cycle 1 before treatment (D-21) after metronomic cyclophosphamide (D1) and after IM treatment (D10), and at the end of IM+IL-2 treatment (D14). No blood sampling was done in subsequent cycles. For each patient, 50 mL of blood were collected in a tube containing heparin and peripheral blood mononuclear cells (PBMCs) were isolated on a Ficoll gradient. Optionally, PBMCs were then frozen in liquid nitrogen until assessment of T-cell and NK-cell abundance, phenotype or function.

Multicolor cytofluorometric studies. PBMC subsets from fresh blood or from thawed vials were analyzed. Cell acquisition and analysis were performed using a FACSCalibur (BD Biosciences) or CyAN (Beckman Coulter) flow cytometer. Data were analyzed with Cell Quest (BD Bioscience) or Flowjo (Tree Star Inc.). Monoclonal antibodies conjugated to fluorescein isothiocyanate (FITC), phycoerythrin (PE), peridinin–chlorophyll–protein complex (PerCP), or allophycocyanin (APC) were used, as follows: MultiTest CD3-FITC/CD8-PE/CD45-PerCP/CD4-APC reagent (342417), MultiTest CD3-FITC/CD16 + 56-PE/CD45-PerCP/CD19-APC reagent (342416), CD3-FITC (345763), CD3-PerCP (345766), CD8-FITC (555366), CD3-PE (34577), CD45RA-FITC (555488), CD27-FITC (340424), HLA-DR-PE (347401), CD69-PE (555531), CCR6-PE (561019), CCR7-PE (552176) (all from BD Biosciences); CD127-FITC (11-1278), PD1-PE (12-9969) CD117-PE (12-1179-73) and TRAIL-PE (12-9927) (all from eBiosciences); CD8-APC (IM2469), CD4-APC (IM2468) and CD56-APC (IM2474) (all from Beckman Coulter); CD25-PE (130-091-024) NKG2D-PE (130-092-672) and NKp44-PE (130-092-480) (all from Miltenyi Biotec); CXCR3-PE (FAB3898A, from IR&D Systems, Inc.); C3XCR1-PE (D070–5, from MBL Co. Ltd). Cell subsets were analyzed after exclusion of doublets and dead cells. Tregs were defined as CD3⁺CD4⁺CD25^{high}CD127^{low} cells and NK cells were defined as CD3⁺CD56⁺ cells. For the detection of NK-cell degranulation and intracellular cytokine production, thawed PBMCs were cocultured with K562 cells (E/T: 10/1) or medium in the presence of a protein transport inhibitor (Golgi-Stop; from BD Biosciences) for 5 h and then stained with ViViD Yellow, CD3-FITC, CD56-PC7 (A21692; from Beckman Coulter),

and—after fixation and permeabilization in Cytofix/Cytoperm (BD Biosciences, 51-2090KZ)—anti-IFN γ -APC (Miltenyi, 130-091-640) plus CD107a-PE (BD Biosciences, 555801).

Statistical analyses. Descriptive data were compared using the χ^2 test or Fisher's exact test for proportions, or the Wilcoxon rank-sum test for continuous measures. Correlation analyses between two parameters were performed by using the Pearson test. OS and PFS were estimated using the Kaplan-Meier method. OS was defined as time from diagnosis to death from any cause or to last follow-up if no death, and PFS was defined as time from diagnosis to progression or to last follow-up if no progression. Patients who had not experienced an event at the time of analysis were censored at the date of last follow-up. Comparisons of PFS and/or OS were performed using Mantel-Cox log-rank tests.

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Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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Supplemental Materials

Supplemental materials may be found here: www.landesbioscience.com/journals/oncoimmunology/article/23080

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