Phase I clinical trial combining imatinib mesylate and IL-2
HLA-DR+ NK cell levels correlate with disease outcome

Nathalie Chaput,1,2,3,4 Caroline Flamant,1,2,4 Clara Locher,1,4,5 Mélanie Desbois,1,2,5 Annie Rey,6 Sylvie Rusakiewicz,1,2,4 Vichnou Poirier-Colame,1,2,4 Patricia Pautier,1,7 Axel Le Cesne,1,7 Jean-Charles Soria,1,5,8 Angelo Paci,1,9 Michelle Rosenzwajg,10,11,12,13 David Klattmann,10,11,12,13 Alexander Eggermont,1,5 Caroline Robert1,7,† and Laurence Zitvogel1,2,5,†

1Institut de Cancérologie Gustave Roussy; Villejuif, France; 2centre d’Investigation Clinique Biothérapie c ICBT 507; Institut de Cancérologie Gustave Roussy; Villejuif, France; 3Unité de Thérapie Cellulaire; Institut de Cancérologie Gustave Roussy; Villejuif, France; 4Institut National de la Santé et de la Recherche Médicale; U1015; Institut de Cancérologie Gustave Roussy; Villejuif, France; 5Faculté de Médecine; Université Paris-Sud; Kremlin Bicêtre, France; 6Service de Biostatistique et d’Épidémiologie; Institut de Cancérologie Gustave Roussy; Villejuif, France; 7Département de Médecine; Institut de Cancérologie Gustave Roussy; Villejuif, France; 8Service Innovations Thérapeutiques Essais Précoces (SITEP); Institut de Cancérologie Gustave Roussy; Villejuif, France; 9Service Interdépartemental de Pharmacologie et d’Analyse du Médicament (SIPAM); Institut de cancérologie Gustave Roussy; Villejuif, France; 10Université Pierre et Marie Curie Université; Paris, France; 11Centre National de la Recherche Scientifique; Unité Mixte de Recherche 7211; Paris, France; 12Institut National de la Santé et de la Recherche Médicale; Unité 5959; Paris, France; 13Clinical Investigation Center in Biotherapy; Hôpital Pitié-Salpêtrière; Paris, France

†These authors contributed equally to this work.

Keywords: cancer, melanoma, NK cells, innate immunity, regulatory T cells, imatinib mesylate, interleukin-2

Abbreviations: AUC, area under the curve; C\text{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 OncoImmunology, 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
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, 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. 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. CTX controls dendritic cell (DC) homeostasis, promotes the secretion of interferon (IFN)α, contributes to the induction of antitumor cytotoxic T lymphocytes (CTLs) and to the proliferation of adaptively transferred T cells, induces the polarization of CD4+ T cells toward Th1 and/or Th17 lymphocytes, and reduced the abundance and functions of regulatory T cells (Treg), eventually favoring tumor regression. 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. IM was first administered to patients with gastrointestinal stromal tumors (GIST) in 2001, 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 PDGFRα (coding for the platelet-derived growth factor receptor α subunit) mutations, with a median progression-free survival (PFS) of 20–24 mo. 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 antitumor cells harboring a hybrid phenotype between DCs and NK cells (MHC Class II+CD11c+CD123+CD56+CD16+CD57+). We have previously reported that IM plus IL-2 was capable of secreting IFNγ 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. The antitumor efficacy of IM plus IL-2 was compromised in mice bearing loss-of-function mutations in the IL-15 receptor α (IL-15Rα) or in Type I IFN receptors 1, and was dependent on plasmacytoid DCs. IL-15Rα 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. 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, and that CTX impairs Treg homeostasis, restoring NK-cell functions, we have conducted a Phase I trial combining CTX, IM and escalating doses of IL-2 (see companion paper published in Oncology 2014), 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 ± 100/mm³), which worsened significantly (p < 0.001) after one cycle of therapy (600 ± 70/mm³). 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/mm³ (R = −0.5255; p = 0.04) (Fig. 1F and 2F). Interestingly, and as already reported in HIV individuals receiving IL-2, the CD4+ : CD8+ T-cell ratio was increased at the end
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 CD56dimHLA-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 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 CD56dim 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 CD56dim 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 IFNγ secretion remained stable (data not shown).
Low doses of IL-2 can activate high-affinity IL-2Rα+CD56bright NK cells, favor NK-cell differentiation from IL-2Rα+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+CD25high 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...
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 OncoImmunology 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 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 CD56dim 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).
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. 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-day 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 CD56bright 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.

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. 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. 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-day 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 CD56bright 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.

![Figure 4.](image)

**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).
as previously described for this cohort of patients,\textsuperscript{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\textsuperscript{+} 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.

In our study, after one cycle of treatment, we could not detect any increase in the number or frequency of CD4\textsuperscript{+} or CD8\textsuperscript{+} T cells (Fig. 1). An increase in CD4\textsuperscript{+}:CD8\textsuperscript{+} 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\textsubscript{H1}/T\textsubscript{H17}-skewed TCR-dependent responses in response to CTX, as previously described for this cohort of patients,\textsuperscript{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\textsuperscript{+} 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.

\textbf{Figure 5.} HLA-DR\textsuperscript{+} natural killer cell levels are positively associated with progression-free and overall survival. (A–D) The median of the relative abundance of HLA-DR\textsuperscript{+} or CD56\textsuperscript{bright} HLA-DR\textsuperscript{+} 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\textsuperscript{+} or high CD56\textsuperscript{bright} HLA-DR\textsuperscript{+} patients (values > median, dotted lines) and low NK HLA-DR\textsuperscript{+} or low CD56\textsuperscript{bright} HLA-DR\textsuperscript{+} 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\textsuperscript{+} NK cells at D10 (A). Curves and p values depicting OS are shown for HLA-DR\textsuperscript{+} NK cells at D10 (B) and D14 (C), as well as for CD56\textsuperscript{bright} HLA-DR\textsuperscript{+} NK cells at D14 (D).
antibodies. Similar findings were obtained in HIV-1-infected patients, who manifested a dramatic increase in CD56bright NK cells. How does IM affect the expansion or activation if 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 CD56bright 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)IFNα2b for 4 weeks in patients with Stage III/IV GIST and reported on the first eight patients who exhibited an objective response. PegIFNα2b promoted a Th1 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. It remains unclear how IL-2 vs. pegIFNα2b 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 CD56bright 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. 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 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 subset of immature NK cells in mice and humans. 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. 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 Biocentre 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 (OncolImmunology 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 Biosciences) 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/CD19-APC reagent (342417), MultiTest CD3-FITC/CD16 + 56-PE/CD45-PercP/CD127-FITC reagent (342416), CD3-FITC (345763), CD3-PerCP (345766), CD8-FITC (555366), CD3-PE (34577), CD45RA-FITC (554588), CD27-FITC (340424), HD-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 BD Biosciences); 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); CXCRI-PE (FAB389A, from IR&D Systems, Inc.); C3XR1-PE (D070–5, from MBL Co. Ltd). Cell subsets were analyzed after exclusion of doublets and dead cells. Tregs were defined as CD3+CD4+CD25highCD127low 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-PE/C7 (A21692; from Beckman Coulter).
and—after fixation and permeabilization in Cytofix/Cytoperem (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 χ² 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.

References

1. Ghiringhelli F, Menard C, Cleris L, Marchesi E, Buchdunger E, Giardini R, et al. In vivo eradication of human BCR/ABL-positive leukemia cells with an ABL kinase inhibitor. Nat Cancer Inst 1999; 91:163-8; PMID:9923858; http://dx.doi.org/10.1093/ canc/91.2.163.

2. Druker BJ, Talpaz M, Buchdunger E, Ohno S, Sagel GM, Fanning S, et al. Effects of a selective inhibitor of the Abl tyrosine kinase on the growth of Bcr-Abl positive cells. Nat Med 1996; 2:356-6; PMID:8616716; http://dx.doi.org/10.1016/0888-7517(96)09056-1.

3. Druker BJ, Talpaz M, Resta DJ, Peng B, Buchdunger E, Ford JM, et al. Efficacy and safety of a specific inhibitor of the BCR-ABL tyrosine kinase in chronic myeloid leukemia. N Engl J Med 2001; 344:1051-7; PMID:11287972; http://dx.doi.org/10.1056/NEJM200104053441404.

4. Buchdunger E, Zimmermann J, Hett M, Tey T, Muller M, Druker BJ, et al. Inhibition of the Abl protein-tyrosine kinase in vitro and in vivo by a 2-phenylaminoypyrimidine derivative. Cancer Res 1996; 56:100-4; PMID:8548747.

5. Joensuu H, Roberts PJ, Sarlomo-Rikala M, Anderson LC, Tervahartiala P, Tivosev D, et al. Effect of the tyrosine kinase inhibitor ST571 in a patient with a metastatic gastrointestinal stromal tumor. N Engl J Med 2003; 349:1054-62; PMID:12387975; http://dx.doi.org/10.1056/NEJM200304033491404.

6. Demetri GD, von Mehren M, Blanke CD, Van den Abbeele AD, Eisenberg B, Roberts PJ, et al. Efficacy and safety of imatinib mesylate in advanced gastrointestinal stromal tumors. N Engl J Med 2002; 347:472-80; PMID:12181401; http://dx.doi.org/10.1056/NEJMoa0204601.

7. Blanke CD, Rankin C, Demetri GD, Ryan CW, von Mehren M, Benjamin RS, et al. Phase III randomized, intergroup trial assessing imatinib mesylate at two dose levels in patients with unresectable or metastatic gastrointestinal stromal tumors expressing the c-kit receptor tyrosine kinase: S0033. J Clin Oncol 2008; 26:2626-32; PMID:18235122; http://dx.doi.org/10.1200/JCO.2007.13.4452.

8. Gastrointestinal Stromal Tumor Meta-analysis Group (MetaGIST). Comparison of two doses of imatinib for the treatment of unresectable or metastatic gastrointestinal stromal tumors: a meta-analysis of 1,640 patients. J Clin Oncol 2010; 28:1247-53; PMID:20121481; http://dx.doi.org/10.1200/JCO.2009.24.2099.

9. Larmonier N, Janikashvili N, LaCasse CJ, Larmonier E, Bindsal C, et al. Imatinib potentiates antitumor T cell responses in gastrointestinal stromal tumor model through the inhibition of IDO. Nat Med 2011; 17:1094-9; PMID:21873999; http://dx.doi.org/10.1038/nm.2419.

10. Shah MH, Freud AG, Benson DM Jr., Ferkitich AK, Dezube BJ, et al. A phase I study of ultra low dose interleukin-2 and stem cell factor in patients with HIV infection or HIV and cancer. Clin Cancer Res 2006; 12:1993-6; PMID:16818697; http://dx.doi.org/10.1158/1078-0432.CCR-06-0268.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

Acknowledgments
This work was supported by Novartis SA, Institut National du Cancer (INCa), la Ligue contre le cancer (LIGUE labellisée, Zitvogel L.), l’Association pour la Recherche sur le Cancer (ARC), Fondation pour la Recherche Médicale, and Fondation de France. The authors thank Lorna Saint Ange (Institut Gustave Roussy) for editing.

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

www.landesbioscience.com
Oncoimmunology
e23080-9
29. Kovacs IA, Lempicki RA, Sidorov IA, Adelsberger JW, Sereti I, Sachau W, et al. Induction of prolonged survival of CD4+ T lymphocytes by intermittent IL-2 therapy in HIV-infected patients. J Clin Invest 2005; 115:2139-48; PMID:16025158; http://dx.doi.org/10.1172/JCI213196.

30. Lévy Y, Thibault R, Gougeon ML, Molina JM, Weiss L, Girard PM, et al.; ILIAD Study Group. Effect of intermittent interleukin-2 therapy on CD4+ T-cell counts following antiretroviral cessation in patients with HIV. AIDS 2012; 26:711-20; PMID:22901410; http://dx.doi.org/10.1097/QAD.0b013e3283519214.

31. Sereti I, Imamichi H, Natarajan V, Imamichi T, Ramchandani MS, Badarulmaa Y, et al. In vivo expansion of CD4CD45RO-CD25 T cells expressing foxP3 in IL-2-treated HIV-infected patients. J Clin Invest 2005; 115:1839-47; PMID:15937547; http://dx.doi.org/10.1172/JCI24307.

32. De Paoli P, Zanussi S, Caggiari L, Bortolin MT, D’Andrea M, Simonelli C, et al. Kinetics of lymphokine production in HIV+ patients treated with highly active antiretroviral therapy and interleukin 2. J Clin Immunol 1999; 19:317-25; PMID:10535609; http://dx.doi.org/10.1023/A:1020547826191.

33. Caligiuri MA, Murray C, Robertson MJ, Wang E, Cochran K, Cameron C, et al. Selective modulation of human natural killer cells in vivo after prolonged infusion of low dose recombinant interleukin 2. J Clin Investig 1993; 91:123-32; PMID:7678599; http://dx.doi.org/10.1172/JCI116161.

34. Hämäläinen EL, Körösföley A, Hadam M, Schneekloth C, Dallmann I, Menzel T, et al. Biological monitoring of low-dose interleukin 2 receptors, cytokines, and cell surface phenotypes. Cancer Res 1993; 51:6312-6; PMID:1933892.