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1 **Pre-transplant donor CD4⁺ invariant NKT cell expansion capacity predicts**
2 **the occurrence of acute graft-versus-host disease**

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44 **Running head:** Graft CD4⁺ invariant NKT expansion predicts GVHD

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46 -Graft CD4⁺ iNKT cell *ex vivo* expansion capacity is the best graft parameter capable of
47 predicting the occurrence of acute GVHD after allogeneic stem cell transplantation from
48 peripheral blood stem cells

49 -Donor CD4⁺ iNKT cell expansion capacity test could be used to select the best donor before
50 performing allogeneic stem cell transplantation with PBSC

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61 **ABSTRACT**

62 Clinically useful pre-transplant predictive factors of acute graft-versus-host-disease
63 (aGVHD) after allogeneic hematopoietic stem cell transplantation (allo-SCT) are lacking. We
64 prospectively analyzed HSC graft content in CD34⁺, NK, conventional T, regulatory T and
65 invariant NKT (iNKT) cells in 117 adult patients before allo-SCT. Results were correlated
66 with occurrence of aGVHD and relapse. In univariate analysis, iNKT cells were the only
67 graft cell populations associated with occurrence of aGVHD. In multivariate analysis, CD4⁻
68 iNKT/T cell frequency could predict grade II-IV aGVHD in bone marrow and peripheral
69 blood stem cell (PBSC) grafts, while CD4⁻ iNKT expansion capacity was predictive in PBSC
70 grafts. ROC analyses determined the CD4⁻ iNKT expansion factor as the best predictive
71 factor of aGVHD. Incidence of grade II-IV aGVHD was reduced in patients receiving a graft
72 with an expansion factor above versus below 6.83 (9.7% vs. 80%, p<0.0001), while relapse
73 incidence at two years was similar (p=0.5).—The test reached 94% sensitivity and 100%
74 specificity in the subgroup of patients transplanted with HLA 10/10 PBSCs without active
75 disease. Analysis of this CD4⁻ iNKT expansion capacity test may represent the first
76 diagnostic tool allowing selection of the best donor to avoid severe aGVHD with preserved
77 GVL effect after PB allo-SCT.

78

79 **INTRODUCTION**

80 Allogeneic hematopoietic stem cell transplantation (allo-SCT) is a curative procedure for
81 malignant haematological disorders, due to the graft versus leukemia (GVL) effect, but is
82 however often hampered by the occurrence of a life threatening acute (a) graft versus host
83 disease (GVHD) (1). Therefore, one of the main challenges of allo-SCT remains to identify
84 factors that would allow predicting the risk of aGVHD without impairing the GVL effect.
85 Although several host and donor pre-transplant clinical, immunological and genetic
86 characteristics have been well established as factors correlated to aGVHD (2), none of them
87 can be used to select the best donor or to help deciding to perform or not SCT.
88 Graft content in T cell subsets has been correlated to the risk of aGVHD, in particular naïve
89 CD45RA⁺ CD4⁺ T cells (3), naïve and central memory CCR7⁺ CD4⁺ T cells (4), or CD8⁺
90 effector memory (CD45RA-CD62L⁻) T cells (5). Immunoregulatory T cells such as
91 CD4⁺CD25⁺FoxP3⁺ CD127^{low} regulatory T lymphocytes (Tregs) (6, 7) and invariant natural
92 killer T (iNKT) cells (8-14) represent other cellular targets in order to regulate donor T cell
93 alloreactivity in experimental mouse models. Some data suggest the involvement of these
94 cells in humans as well (15-22). We previously reported that enhanced early post-transplant
95 iNKT cell reconstitution from donor cells was correlated to reduced risk of aGVHD without
96 impairment of the GVL effect (23) while Treg cell reconstitution was not. Likewise, graft
97 content in CD4⁻ iNKT cells, but not in Tregs, was associated to the risk of aGVHD in allo-
98 SCT performed with peripheral blood stem cells (PBSCs) from HLA-identical siblings (24,
99 25). Although these studies suggest a role of CD4⁻ iNKT cells for the control and prediction
100 of aGVHD, because of their great variability in terms of numbers, it is currently difficult to
101 define a threshold of graft iNKT cell dose predicting with high specificity and sensitivity the
102 risk of aGVHD. We show here that *in vitro* donor CD4⁻ iNKT cell expansion capacity
103 represents the best predictive factor of aGVHD without impairment of the GVL effect in allo-
104 SCT performed with PBSC grafts.

105

106 PATIENTS AND METHODS

107 Patients

108 Between June 2010 and January 2014, 117 adult patients who underwent an allo-SCT in
109 three transplant institutions (Saint Antoine, Necker and Pitié-Salpêtrière hospitals, Paris)
110 entered the study. All patients and their sibling donors provided an informed consent for
111 biological research purposes. The study was conducted according to the procedures of the
112 Declaration of Helsinki and the local ethic committee rules. Diagnosis and grading of acute
113 and chronic GVHD were performed as described (26, 27) by physicians in charge of the
114 patients who were not aware of the results of laboratory tests.

115 Flow cytometry analysis

116 Analyses were performed on 117 HSC grafts (freshly harvested in 96 and from thawed cells
117 in 21). Among those, 21 freshly harvested sibling donor blood cells drawn before
118 mobilization of HSC by G-CSF were analyzed and compared to the corresponding HSC
119 graft. Graft cell phenotype was performed in all 117 HSC grafts, while *ex vivo* iNKT
120 expansion tests could only be performed on the 96 freshly harvested grafts. Post-transplant
121 peripheral immune reconstitution was analyzed from 30 recipients on days 30±5, 60±5 and
122 90±5 days after transplantation (**Figure S1**). In all analyses, mononuclear cells were isolated
123 by density-gradient centrifugation (Ficoll-Paque™ PLUS, GE Helthcare). Cells were first
124 stained with PBS57-loaded or empty-CD1d-tetramers (NIH Tetramer Core Facility) and with
125 the following monoclonal antibodies: anti-CD3, -CD4, -CD8, -CD56, -CD25, -CD127, -
126 CD45RA, - $\gamma\delta$ TCR, -CD161, -FoxP3, -Ki67 (eBioscience), anti-V α 7.2TCR (Biolegend). Data
127 were acquired on a FACS Canto II flow cytometer using FACS Diva software (BD
128 Biosciences), and were analyzed with the FlowJo software (Tree Star Inc). Lymphocyte
129 subpopulations were analyzed within the lymphocyte gate on forward and side-scatter plots
130 as described in **Figure S2**. iNKT cells were specifically detected by the co-expression of
131 CD3 and PBS57-loaded CD1d-tetramer, Tregs were analyzed as described by Sakaguchi's

132 group (28) and Mucosal-associated invariant T (MAIT) cells were identified as previously
133 reported (29). Results were expressed in absolute numbers or in terms of cell subtype/ 10^2 or
134 10^3 T lymphocyte ratios (**Table S1**). Since iNKT cells represent a rare population of T
135 lymphocytes, data were considered as reliable when a minimum of 1×10^6 mononuclear cells
136 were stained and at least 5×10^4 CD3⁺ T cells with a minimum of 20 iNKT CD3⁺ CD1d
137 Tetramer⁺ cells acquired. Doses of the different cell subtypes were extrapolated from the
138 multiplication of CD3⁺ T cell dose by the corresponding cell subtype/T cell ratio.

139 **Functional studies of iNKT cells**

140 Mononuclear cells from freshly harvested peripheral blood, BM or PBSC grafts were
141 cultured in 24-well plates at a density of 1×10^6 cells per well in 2mL of RPMI-1640 medium
142 supplemented with 10% fetal bovine serum (FBS), 100U/mL penicillin and streptomycin,
143 200mM L-glutamine, and 10mM HEPES (All from Life Technologies). Alpha-
144 galactosylceramide (KRN7000, Avanti Polar Lipids) was added at 100 ng/mL at the onset of
145 culture, followed 24h later by rhIL-2 at 845U/mL (ImmunoTools). After 2 weeks, cells were
146 collected, washed extensively before surface staining, as described above and represented in
147 **Figure S3**. The expansion factor was calculated by dividing the number of each cell subset
148 obtained on Day 15 by the number of cells on Day 0.

149 **Statistical analyses**

150 Statistical analyses were performed using the R program (30). Means were compared using
151 the non-parametric Wilcoxon and proportions using the Fisher exact or Chi-square tests, as
152 appropriate. Correlations between continuous variables were calculated using the non-
153 parametric Spearman's rank correlation test. Overall survival (OS) was measured from the
154 date of HSCT to the date of death from any cause or of last follow-up. Non-relapse mortality
155 (NRM) was defined as death occurring in continuous complete remission (CR). None of the
156 patients were lost of follow-up and all were censored at date of death or last-follow-up.
157 Median follow up of patients alive was 681 days (range 153-1348).

158 Univariate and multivariate Cox proportional hazards models were analyzed using the
159 survival package (31). OS curves were established using the Kaplan-Meier method and
160 statistical differences in survival distributions according to baseline parameters and the
161 expansion factor of CD4⁺ iNKT cells were assessed using the logrank test. Cox proportional
162 hazard model was used for multivariate analysis. CD4⁺ iNKT/10³ T frequency and CD4⁺
163 iNKT expansion factor covariates were log transformed to ensure a normal distribution. The
164 martingale residuals versus the log-transformed covariates were well distributed around the 0
165 ordinate line, with no trend or particular shape observed. Acute GVHD occurred in all
166 patients before day 100 and was analyzed as a time-dependent covariate in the Cox model.
167 Stepwise regression consisting of alternating forward and backward elimination steps was
168 used to find the most parsimonious set of significant covariates using the Bayesian
169 Information criterion (BIC). The internal validation of the multivariate analysis was assessed
170 using a bootstrap method.

171 In order to assess the ability of the expansion factor of CD4⁺ iNKT cells to predict aGVHD,
172 the value of that factor and actual development of aGVHD were subjected to receiver
173 operating characteristic (ROC) curve analysis (32) using the ROCR package (33). A
174 bootstrap method was used to calculate the 95% confidence interval of the AUC of ROC
175 curves.

176 A p-value of 0.05 was considered statistically significant for all analyses, except for
177 univariate analysis including multiple biological parameters associated with the occurrence of
178 acute GVHD in which an adjusted p-value of 0.0017, corresponding to 0.05/30 factors, was
179 applied. Two-sided tests were used in all analyses.

180

181 RESULTS

182 Patients, treatment assignments and transplant outcomes

183 Clinical characteristics for the 117 patients included in the study are summarized in **Table 1**.

184 Median age at transplantation was 49.7 years for recipients and 42.4 years for donors. A
185 majority of patients (66%) received an allogeneic HSCT for acute leukemia. Sixty nine
186 (59%) received an allograft from an HLA-identical sibling, 42 (36%) from a matched
187 unrelated donor and 6 (5%) from a mismatched unrelated donor. HSC were obtained from
188 unmanipulated BM in 33 (28%) or PBSC in 84 (72%) transplanted patients. The choice of
189 conditioning was dependent on the underlying disease, recipient's age and co-morbidities:
190 myeloablative conditioning (MAC) for patients in CR under 50 years of age and without co-
191 morbidities (42%), conventional reduced intensity conditioning (RIC) for patients above 50
192 years or presenting co-morbidities (47%), and sequential-based RIC regimen (34) for
193 refractory diseases (11%). Altogether, 63 (54 %) patients received an *in vivo* T cell depletion
194 with ATG-Thymoglobuline (total dose 5 mg/kg), mainly with unrelated PBSCs or with
195 fludarabine-busulfan RIC.

196 With a median follow up of 15 months (range 1-45), cumulative incidence (CI) of grade II-
197 IV aGVHD was 25% (95% CI, 14-38%) at day 100. At 2 years, CI of NRM was 14.5% (95%
198 CI, 3-35%), CI of chronic GVHD was in 57% (95% CI, 47-66%), relapse incidence was 35%
199 (95% CI, 22-48%) and OS was 64 % (95% CI, 53-73%).

200 Graft cell content and iNKT subsets' expansion capacities of donor grafts

201 In all 117 HSC grafts, we measured graft cell content in CD34, CD56 NK, CD3 T, total CD4
202 T, CD45RA⁺ and CD45RA⁻ CD4 T, CD8 T, $\gamma\delta$ T, MAIT, total, naïve and memory Tregs, and
203 total iNKT cells, as well as CD4⁺ and CD4⁻ iNKT cell subsets. CD4⁺ and CD4⁻ iNKT cell
204 expansion was carried out in 96 freshly harvested grafts (77 from PB and 19 from BM stem
205 cells) (**Table S1**). The frequencies of total iNKT and CD4⁻ iNKT cells among T cells were
206 similar between PBSC and BM grafts, while the frequency of the minority CD4⁺ iNKT cell

207 subtype was slightly higher in BM than in PBSC grafts ($p=0.02$) (**Table S2**). Because PBSC
208 contained one log higher number of T cells than BM grafts, all iNKT cell subset doses were
209 increased in PBSCs ($p<0.0001$) (**Table S2**). *Ex vivo* expansion capacities of both CD4⁺ and
210 CD4⁻ iNKT cells were higher in PBSC as compared to BM grafts ($p<0.05$) (**Table S2**). Age
211 of donor had a negative impact on the frequency and dose of CD4⁺ iNKT cells ($p=0.049$), but
212 not on CD4⁻ iNKT cells (**Table S3**).

213 **Graft parameter and pre-transplant factors associated with the occurrence of grade II-** 214 **IV aGVHD**

215 In univariate analysis, in the total cohort of patients, those who developed grade II-IV
216 aGVHD received a graft with higher frequencies of CD45RA⁺ CD4⁺ T cells ($p=0.045$), lower
217 frequencies of total, CD4⁺ and CD4⁻ iNKT cells ($p=0.001$, $p=0.021$ and $p=0.0005$,
218 respectively), and lower doses of total and CD4⁻ iNKT cells ($p=0.027$ and 0.025 ,
219 respectively) (**Table 2 and Figure S4**). Functional analyses of iNKT cell subsets showed
220 significantly reduced expansion capacities of the CD4⁻ iNKT cells in patients developing
221 grade II-IV aGVHD ($p=0.0007$). None of the other graft parameters analyzed including total,
222 naïve and memory Tregs were associated with the occurrence of acute GVHD. The only pre-
223 transplant parameter associated with a higher risk of developing grade II-IV aGVHD was the
224 use of an unrelated donor ($p= 0.00004$) (**Table 2**).

225 Cumulative incidence of grade II-IV aGVHD at day 100 in patients receiving a graft with an
226 iNKT cell dose below or above the median for total iNKT cells was 33% versus 17%
227 ($p=0.028$). However, when p-value was adjusted to the multiple tests performed in univariate
228 analysis, the remaining significant factors associated to the occurrence of aGVHD were
229 frequencies of total iNKT cells, frequency and expansion capacity of CD4⁻ iNKT cells and
230 type of donor ($p\leq 0.00017$).

231 Analyses performed separately between patients transplanted with BM and PB confirmed the
232 correlation between frequencies of total and CD4⁻ iNKT cells and occurrence of aGVHD in

233 both subgroups of stem cell source, but the association with the CD4⁺ iNKT expansion factor
234 was only observed in the PBSC group (Tables S3 and S4).

235 In multivariate analysis, among all the graft parameters analyzed, the only two associated
236 with an independent reduced risk of developing grade II-IV aGVHD were the frequency of
237 CD4⁺ iNKT cells (Odd ratio (OR)=0.56, 95% confidence interval (CI)= 0.38-0.84, p=0.0048)
238 and the expansion factor of CD4⁺ iNKT cells (OR=0.72, 95% CI= 0.55-0.82, p<0.001)
239 (**Table 3, Model 1**). The use of matched unrelated donors (MUD) and progressive disease
240 (PD) status at transplant were the other pre-transplant factors associated with an increased
241 risk of grade II-IV aGVHD (OR=8.16, 95% CI=3-22.1, p<0.0001 for MUD and OR=10, 95%
242 CI=3-33.5, p=0.00017 for PD) (**Table 3, Model 1**). Predictive value of these factors on the
243 occurrence of severe aGVHD was confirmed in an internal validation test by using the
244 bootstrap method (**Table 3**).

245 In a Cox model taking into account the stem cell source and after adjusting to donor type and
246 disease status at transplantation, the graft CD4⁺ iNKT/T frequency could significantly predict
247 the occurrence of grade II-IV aGVHD in both PB and BM transplants, but the CD4⁺ iNKT
248 expansion factor was only significant in PBSC grafts (**Table 3, Model 2**).

249 **Determination of the best graft factor and threshold predicting aGVHD occurrence**

250 Using ROC analyses, the predictive value in the global population of patients with available
251 iNKT expansion tests of the whole model including CD4⁺ iNKT frequency and expansion
252 factor, donor type and disease status at SCT was high with an area under the curve (AUC) of
253 0.927 (95% CI: 0.86-0.994) (**Figure 1A**). However, because such model remains complex for
254 clinical application, we sought to determine which of the graft CD4⁺ iNKT frequency or
255 expansion factor might be the most useful. In this objective, we compared the sensitivity and
256 specificity of these two parameters to predict the occurrence of grade II-IV aGVHD in the
257 total population of patients and in the subgroups of patients transplanted with BM or PB stem

258 cells (**Table 4A**). Both factors were highly sensitive (>90%) but the CD4⁺ iNKT expansion
259 factor provided the best specificity in all subpopulations of patients analyzed.

260 In the total population of 96 patients with available functional iNKT tests, the CD4⁺ iNKT
261 expansion factor could discriminate the risk of grade II-IV aGVHD at day 100, with an AUC
262 of 0.767 (**Table 4A and Figure 1B**). A CD4⁺ iNKT expansion factor cut-off of 6.83 provided
263 the best balance between sensitivity and specificity, with 94% true and 36% false positive
264 rates. Pre-transplant characteristics of patients transplanted with a graft providing a CD4⁺
265 iNKT expansion factor below or above the 6.83 threshold were comparable except for
266 increased siblings in the good expander group (73.5% vs 30%, p=0.00037) (**Table S6**). Using
267 this cut-off point, 16 patients out of 20 (80%) with a CD4⁺ iNKT expansion factor below 6.83
268 experienced grade II-IV aGVHD, while only 9 out of 76 (12%) with CD4⁺ iNKT expansion
269 factor above 6.83 did so (**Figure 1C**). In contrast, relapse incidence at 2 years was
270 comparable between the two groups (31% vs. 35%, p=0.50) (**Figure 1D**), as well as
271 incidence of chronic GVHD (CI at 2 years of 68% vs. 55%, p=0.45) (**Figure 1E**). In this
272 series of patients, 25 developed a grade II and 4 a grade III-IV aGVHD, of whom only 4
273 patients died of acute and 2 of chronic GVHD. At two years, NRM and OS therefore were
274 comparable between the two groups of patients (17.2% versus 10.4%, p=0.53, and 67%
275 versus 63%, p=0.58, respectively) (**Figure 1F and 1G**, respectively).

276 In the subgroup of patients transplanted with PBSC grafts with available iNKT expansion
277 tests (n=77), the AUC of the expansion test was 0.772 (**Table 4A**). Using an expansion factor
278 cutoff of 8.7, 12 of 15 (80%) patients with an expansion factor below 8.7 developed grade II-
279 IV aGVHD compared to 6 of 62 (10%) patients above the threshold (p<0.001). These two
280 subgroups of patients had otherwise comparable relapse risk, non relapse mortality and
281 overall survival (data not shown).

282

283 **Correlation between graft CD4⁻ iNKT expansion factor, other graft characteristics and**
284 **post-transplant iNKT reconstitution**

285 We compared the different graft characteristics in patients who received a graft with a CD4⁻
286 iNKT cell expansion factor above (n=76) or below (n=20) the previously determined
287 threshold of 6.83 in the total population of patients. The CD4⁺ iNKT cell expansion factor
288 was also higher in grafts with a CD4⁻ iNKT cell expansion factor ≥ 6.83 in comparison to
289 those below that threshold (p=0.03) (**Figure 2A**). Grafts with a higher expansion capacity of
290 CD4⁻ iNKT cells contained similar doses of CD34⁺ HSC, NK cells, CD3⁺, CD4⁺, CD8⁺, $\gamma\delta^+$
291 and regulatory T cells than grafts with a lower expansion capacity but higher doses of MAIT
292 cells (p=0.01) (**Figure 2B**). Grafts with enhanced CD4⁻ iNKT expansion capacity had also
293 significantly higher frequencies and doses of total and CD4⁻ iNKT cells (p<0.05 for all)
294 (**Figure 2C and 2D**). Patients who received a graft with a CD4⁻ iNKT cell expansion factor
295 above 6.83 (n=21) had higher levels of total PB iNKT cells on day 30, 60 and day 90 (p<0.05
296 for all) post-transplantation than those with a CD4⁻ iNKT expansion factor < 6.83 (n=9)
297 (**Figure 2E**).

298 **Determination of the most appropriate allo-SCT context in which the CD4⁻ iNKT**
299 **expansion test can predict aGVHD**

300 Since occurrence of grade II-IV aGVHD was also associated with progressive disease status
301 at transplant (**Table 3**), we performed ROC analyses in the 79 patients allografted in non-
302 progressive disease with an HLA 10/10 matched donor. In comparison to the whole
303 population, the value of the graft CD4⁻ iNKT expansion factor AUC in the prediction of
304 grade II-IV aGVHD was improved in this subgroup of patients (AUC=0.90) (**Table 4B**). In
305 this context, the predictive value of the test was confirmed to be limited for BM grafts
306 (AUC=0.679) but optimal for PBSC grafts (AUC=0.986), with 94% sensitivity and 100%
307 specificity (**Table 4B and Figure 3**). In the predominant group of patients transplanted with
308 HLA compatible PBSCs and non progressive disease at transplant (n=60), ten of the 14

309 patients (71%) who received a graft with a CD4⁺ iNKT expansion factor below the threshold
310 of 8.7 developed grade II-IV aGVHD, while none of the 46 patients having received a graft
311 with a CD4⁺ iNKT expansion factor above that threshold did so ($p < 0.0001$) (**Figure 3**).
312 We finally compared the CD4⁺ iNKT cell expansion factor from the HSC graft to that
313 obtained from PBMC from the same donor before the administration of G-CSF (**Table S7**).
314 Although the expansion factor was often higher in the graft, the two tests could similarly
315 predict the risk of occurrence of aGVHD in all 21 cases tested.
316

317 **DISCUSSION**

318 This study confirms the value of iNKT cells as predictors of aGVHD after allo-SCT in
319 humans. As reported in the context of allo-SCT performed with PBSC from HLA-identical
320 sibling donors without *in vivo* T cell depletion (25), our study confirms, that the HSC graft
321 content in total and CD4⁺ iNKT cells represents the only graft cell subtype associated with the
322 occurrence of grade II-IV aGVHD. In multivariate analyses, we show that higher graft CD4⁺
323 iNKT T frequencies are associated with reduced risk of aGVHD in BM grafts, while both
324 graft CD4⁺ iNKT frequency and expansion factor could independently predict the occurrence
325 of aGVHD in PBSC grafts. ROC curves show that the *ex vivo* expansion factor of CD4⁺
326 iNKT cells represents the best predictive factor for the occurrence of aGVHD in allo-SCT
327 performed with PBSCs. This iNKT functional test reaches its optimal predictive capacity
328 with 100% specificity in allo-SCT performed with HLA 10/10 matched PBSC grafts for non-
329 progressive hematological malignant diseases, which represents the majority of the
330 indications of allogeneic SCT. Similar predictive value was also observed when the test was
331 performed from donor's peripheral blood before G-CSF mobilization. Thus, this test could
332 allow to easily select the best donor if different siblings or unrelated donors are available
333 before PB allo-SCT.

334 In line with the study of Chaidos et al. who reported, in allo-SCT performed without ATG, a
335 reduced incidence of aGVHD at day 180 post-transplant in the group of patients receiving a
336 dose of total iNKT cells above the median (31% versus 61%, p=0.014) (25), we observed
337 reduced incidence of aGVHD at day 100 post-transplant in patients receiving a total dose of
338 iNKT cells above the median (17% versus 33%, p=0.028). These observations contrast with
339 the study of Malard et al., who did not find a significant correlation between iNKT cell dose
340 and aGVHD incidence in a series of 80 RIC allo-SCT performed with PBSC and ATG (20%
341 versus 25%, above and below the total iNKT median dose, respectively, p=0.53) (35). In
342 contrast with other T cells, iNKT cells are spared by the use of total lymphoid irradiation

343 (TLI) and ATG in mice and humans (36-38). Thus, iNKT/T cell frequencies seem more
344 meaningful to be analyzed, in particular in the context of clinical use of ATG. Unfortunately,
345 such analysis has not been performed in Malard's study.

346 As described by others (39), the proportions of iNKT cells after G-CSF mobilization were
347 similar between PBSC and BM grafts. However, iNKT cell doses were significantly
348 increased in PBSC, and G-CSF mobilized iNKT cells were more prompt to expand in
349 response to rhIL-2 and α -GalCer, possibly due to reduced antigen presenting cell numbers in
350 BM versus PB. This may explain the absence of predictive value of the CD4⁻ iNKT cell
351 expansion factor from BM grafts on the occurrence of aGVHD. We also observed that
352 frequencies, doses and expansion capacity of CD4⁻ iNKT cells were not correlated with
353 donor age, whereas numbers of CD4⁺ iNKT cells declined with donor's age, a phenomenon
354 explained by the dependence of CD4⁺ iNKT cells on thymic output, while CD4⁻ iNKT cells
355 can undergo peripheral homeostatic expansion (40, 41).

356 Although the frequency of iNKT cells and their *ex vivo* expansion capacity in the presence of
357 IL-2 and α -GalCer are known to be very variable between individuals (40-42), the reasons
358 are poorly understood. Croudace et al. showed that poor proliferative responses of iNKT cells
359 to IL-2 and α -GalCer were not dependent on the type and concentration of the CD1d ligand
360 used, nor to the disappearance of iNKT cells during the 14-day culture period, but could be
361 partially salvaged by the addition of IL-4 (42). The expansion factor of CD4⁺ iNKT cells was
362 higher in grafts with a CD4⁻ iNKT expansion factor above 6.83 in comparison to those below
363 that threshold. Thus, the difference of CD4⁻ iNKT cell expansion in the presence of rhIL-2
364 and α -GalCer in healthy donors might be due to distinct CD4⁺ iNKT cells response to IL-2,
365 which might subsequently, directly or indirectly impact on the expansion of to the CD4⁻
366 iNKT cell subset. It has been shown that murine iNKT cells undergo a bystander expansion
367 and activation depending on IL-2 produced by alloreactive T cells after allo-SCT (43). We
368 observed an enhanced early post-transplant iNKT cell recovery in patients who received a

369 graft with higher *ex vivo* CD4⁻ iNKT cell expansion capacities. These data confirm our
370 previous report correlating post-transplant iNKT cell recovery with the occurrence of
371 aGVHD (23), and suggest that the *ex vivo* expansion test of CD4⁻ iNKT cells can reflect and
372 predict the *in vivo* expansion capacities of these cells.

373 In our study, grade II-IV aGVHD occurred in 25 patients and 4 of them developed grade III-
374 IV aGVHD, which was lethal in 2 cases. Although the CD4⁻ iNKT cell expansion factor
375 could highly predict the occurrence of aGVHD, no impact on NRM and OS was observed.
376 Despite a very low risk of aGVHD, patients who received a graft with a CD4⁻ iNKT cell
377 expansion factor above 6.83 had preserved GVL effect without increased relapse risk,
378 possibly due to the unaffected development of chronic GVHD. This observation was in line
379 with preserved GVL effect observed in patients with higher iNKT cell recovery after allo-
380 SCT in other studies (21, 23).

381 The mechanisms by which human CD4⁻ iNKT cells can control GVHD while preserving the
382 GVL effect remain to be determined. By comparison to CD4⁺ iNKT cells, their CD4⁻
383 counterparts kill dendritic cells (DCs) and reduce the maturation of monocyte-derived DCs
384 (25, 44). Whether they can induce Tregs or modulate the cytokine orientation of allogeneic T
385 cell responses, as reported for murine iNKT cells in mouse models of GVHD (37, 45), will
386 require further studies.

387 In conclusion, despite the limitations due to the heterogeneity of our series of patients and the
388 need for prospective validation studies, we describe a clinically useful and relevant tool that
389 could predict at the individual level the occurrence of aGVHD without impairment of the
390 GVL effect after allo-SCT performed with PBSC grafts. Therefore, this test could easily be
391 used to select the best donor before transplantation and/or to adapt post-transplant
392 immunosuppressive treatments in the recipient.

393

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405 **Authorship contributions:**

406 *Designed the study: MTR, MB, OH*

407 *Collected the data: MTR, MB, TC, FS, DS, AB*

408 *Provided patients data: MTR, FS, AG, DS, AB, EP, SNK, MM, OH*

409 *Provided materials and expertise in cell phenotyping: HTN, MC, OL*

410 *Analyzed the data: MTR, MB, NB, SU, OH*

411 *Wrote and commented on the manuscript: MTR, MB, MM, NB, SU, OH*

412 *All authors approve the final manuscript*

413 **Disclosure of Conflict of interest**

414 The authors declare no financial conflict of interest.

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536

537 **Figure legends**

538 **Figure 1: ROC analysis and according to the expansion factor of CD4⁺ iNKT cells**

539 (A) ROC analysis of the whole model including donor type, disease status at transplantation, graft
540 CD4⁺ iNKT/T frequency and expansion factor in predicting the occurrence of grade II-IV acute
541 GVHD in the total population of patients (B) ROC analysis of the expansion factor of CD4⁺ iNKT
542 cells in predicting the occurrence of grade II-IV acute GVHD in the total population of patients.
543 Choice of the 6.83 cut-off point determining the best sensitivity and specificity (94% true positive rate
544 and 36% false positive rate. Cumulative incidence of (C) grade II-IV acute GVHD, (D) relapse, (E)
545 chronic GVHD, (F) non relapse mortality (NRM), and (G) probability of overall survival (OS)
546 according to the CD4⁺ iNKT expansion factor (<6.83 in dashed line and ≥ 6.83 in continuous line) in
547 the total population of patients. Comparisons are performed with the log-rank test.

548 **Figure 2: Graft content and post-transplant iNKT recovery according to the graft CD4⁺**
549 **iNKT expansion factor**

550 Graft characteristics and post-transplant iNKT reconstitution were analyzed in two groups according
551 to their graft CD4⁺ iNKT expansion factor (exp.fact.): ≥ 6.83 (grey) or < 6.83 (white) (A) Graft CD4⁺
552 iNKT expansion factor, (B) Graft cell subset frequencies, (C) Graft iNKT cell subset frequencies, and
553 (D) Graft iNKT cell subset doses. Results are presented as mean ± SEM. (E) Recipient peripheral
554 blood levels of total iNKT cells on days 30, 60 and 90 post-transplantation according to CD4⁺ iNKT
555 expansion factor. Means were compared with unpaired t test. ns=non significant.

556 **Figure 3: ROC analysis and cumulative incidence of grade II-IV aGVHD in the**
557 **subgroup of patients allografted at non-progressive disease with an HLA 10/10 PBSC**
558 **graft**

559 (A) ROC analysis of the expansion factor of CD4⁺ iNKT cells in predicting the occurrence of grade II-
560 IV acute GVHD in the subpopulation of patients allografted with non-progressive disease with an
561 HLA 10/10 PBSC graft (n=60, AUC=0.986) Determination of the 8.7 cut-off point providing the best
562 sensitivity and specificity (94% true positive rate and 0% false positive rate). (B) Cumulative
563 incidence of grade II-IV acute GVHD according to the CD4⁺ iNKT expansion factor in this subgroup
564 of patients (<8.7 in dashed line, and ≥ 8.7 in continuous line). Comparisons are performed with the
565 Log-rank test.
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567 **Tables**

568

569 **Table 1. Patients' characteristics at transplant**

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Baseline parameters	All patients
All patients (numbers, %)	117 (100%)
Gender	
Male	76 (65 %)
Female	41 (35 %)
Age of recipient (years)	
Median (range)	49.7 (16.5-69)
Age of donor (years)	
Median (range)	42.4 (18.2-66.4)
Hematological disease	
AML	58
ALL	19
MPD	6
MDS	11
Lymphoma	15
Myeloma	7
AA	1
Disease status at transplant	
CR	68 (58 %)
PR or SD	36 (31 %)
PD	13 (11%)
Donor	
Sibling	69 (59 %)
Unrelated 10/10 HLA-matched	42 (36 %)
Unrelated 9/10 HLA-matched	6 (5 %)
Donor/recipient gender matching	
Female to male	27 (23 %)
other	90 (77 %)
HSC source	
Bone marrow	33 (28%)
Peripheral blood	84 (72 %)
Conditioning regimen	
MAC	49 (42%)
RIC	68 (58%)
In vivo T-cell depletion	
No	54 (46 %)
Yes	63 (54 %)
GVHD prophylaxis regimen	
CsA + MTX	49 (42 %)
CsA + MMF	52 (44.5 %)
CsA alone	16 (13.7 %)
CMV risk	
Low	24 (20.5 %)
Intermediate	67 (57.3 %)
High	26 (22.2 %)

AA: aplastic anemia, AML: acute myeloid leukemia, ALL: acute lymphoid leukemia, CsA: cyclosporine A, MDS: myelodysplastic syndrome, MMF: mycophenolate mofetil, MPD: myeloproliferative disease, MTX: methotrexate, AA: aplastic anemia, CR: complete remission, PR: partial remission, PD: progressive disease, MAC: myeloablative conditioning, RIC: reduced intensity conditioning, HSC: hematopoietic stem cells, PBSC: peripheral blood stem cells. CMV reactivation risks were determined according to the donor (D) and recipient (R) serology as follows: D-/R-: low risk, D+/R+ or D+/R-: intermediate risk, D-/R+: high risk.

Table 2: Univariate analysis of parameters associated with the development of a GVHD

Parameter	aGVHD 0-I		aGVHD II-IV		p-value ^ω
	Mean	SD	Mean	SD	
Graft cell subset frequencies (cell subset number/10³ T-cells)					
CD4 ⁺ T	611.59	110.5	599.29	98.13	0.582
CD45RA⁺CD4⁺ T	277.13	101.6	314.73	96.71	0.045
CD45RA ⁻ CD4 ⁺ T	329.37	89.8	284.56	91.52	0.089
CD8 ⁺ T	367.3	95.5	371.42	82.93	0.747
γδ ⁺ T	68.01	88.8	76.19	78.9	0.308
T regs	12.13	9.1	9.6	7.14	0.313
Naïve T regs	2.85	4.3	2.34	2.63	0.863
Memory T regs	9.37	6.6	7.46	6.11	0.192
NK	117.07	165.2	94.48	55.94	0.834
MAIT	29.07	27.5	21.92	13.62	0.429
Total iNKT	1.94	2.6	0.87	1.06	0.0016
CD4⁺ iNKT	0.56	1	0.32	0.47	0.021
CD4⁻ iNKT	1.38	1.8	0.55	0.78	0.00059
Graft cell subset doses (x10⁶/kg)					
CD34 ⁺	4.96	2.3	4.53	2.57	0.317
CD3 ⁺ T	170.2	134	202.5	151	0.263
CD4 ⁺ T	103.73	87.7	119.68	94.09	0.315
CD45RA⁺CD4⁺ T	48.96	45.2	67.22	57.51	0.193
CD45RA ⁻ CD4 ⁺ T	55.84	48.7	55.25	42.84	0.704
CD8 ⁺ T	62.67	56.6	72.01	59.83	0.579
γδ ⁺ T	15.9	28.8	21.2	69.86	0.782
T regs	1.93	2.1	2.07	2.55	0.722
Naïve T regs	0.4	0.7	0.52	0.94	0.919
Memory T regs	1.56	1.7	1.55	1.88	0.679
NK	19.61	31.9	18.43	14.59	0.519
MAIT	4.5	6.8	4.88	6.41	0.789
Total iNKT	0.27	0.4	0.17	0.29	0.027
CD4 ⁺ iNKT	0.09	0.2	0.05	0.08	0.066
CD4⁻ iNKT	0.2	0.3	0.12	0.24	0.025
Expansion factor of iNKT cell subsets					
CD4 ⁺ iNKT expansion factor	471.81	614.8	366.55	648.98	0.07537
CD4⁻ iNKT expansion factor	88.71	104.1	36.32	63.31	0.00007
Pre-transplant clinical factors					
Recipient age	47.56	14.5	45.99	13.73	0.525
Donor age	42.31	13.5	38.09	12.18	0.118
	Nb	%	Nb	%	p-value
Disease status at HSCT					0.085 ^χ
CR/PR/SD*	81	92.1	23	79.3	
PD	7	7.9	6	20.7	
Donor type					0.00004^ψ
HLA identical sibling	62	70.5	7	24.1	
Matched unrelated	23	26.1	19	65.6	
Mismatch unrelated	3	3.4	3	10.3	
Female to male					
HSC source					0.813 ^χ
PBSC	64	72.7	20	69	
Bone marrow	24	27.3	9	31	
Conditioning regimen					1 ^χ
MAC	37	42	12	41.4	
RIC	51	58	17	58.6	
In vivo T cell depletion					1 ^χ
Yes	47	53.4	16	55.2	
No	41	46.6	13	44.8	
CMV risk					0.799 ^χ
High	19	21.6	7	24.1	
Other	69	78.4	22	75.9	

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aGVHD: acute GVHD, iNKT: invariant NKT cells, CR: complete remission, HSC: hematopoietic stem cells, MAC: myeloablative conditioning, MAIT: mucosa associated invariant T cells, PBSC: peripheral blood stem cells, , PD: progressive disease, PR: partial remission, RIC: reduced intensity conditioning, D: standard deviation, SD*: stable disease ,T regs: regulatory T cells, Ψ: fisher exact test, χ: chi2 test, ω: Wilcoxon test.

625 **Table 3. Cox analysis and internal validation test of predictive factors of grade II-IV**
 626 **acute GVHD development**

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Baseline parameters	Multivariate analysis [§]			Bootstrap analysis
	HR	95% CI	p [#]	Median [95% CI]
Model 1 (all grafts)				
Donor type				
HLA identical sibling	1			
Matched unrelated	8.16	3 -22.1	3x10⁻⁵	16.9 [4.54 – 187]
Mismatched unrelated	3.2	0.68-15.5	0.14	4.9 [0 –695]
Disease status at transplant				
CR/PR or SD	1			
PD	10	3-33.5	0.00017	18.5 [4.1 – 199]
Stem cell source				
PB	1			
BM	2.61	0.93-7.33	0.068	1.62 [0.33 – 7.9]
CD4⁺ iNKT/10³ T frequency				
	0.56	0.38-0.84	0.0048	0.49 [0.23 – 0.79]
CD4⁺ iNKT expansion factor				
	0.72	0.55-0.82	0.00013	0.65 [0.49 – 0.82]
Model 2*				
CD4⁺ iNKT/10³ T frequency				
PB	0.66	0.49-0.89	0.0005	
BM	0.47	0.26-0.84	0.01	
CD4⁺ iNKT expansion factor				
PB	0.59	0.46-0.75	1.94x10⁻⁵	
BM	0.81	0.53-1.24	0.34	

628 *BM: bone marrow, CI: confidence interval, CR: complete remission, HR: hazard ratio, NA: non applicable, PB: peripheral*
 629 *blood, PD: progressive disease, PR: partial remission, SD: stable disease, , #Time dependant Cox model, § Variables*
 630 *included in the Cox model were: HSC source, donor type, donor and recipient age, disease status at transplant, use of in*
 631 *vivo T cell depletion, and the following graft parameters: total iNKT/10³ T, total iNKTx10⁶/kg, CD4⁺ iNKT/10³ T, CD4⁺*
 632 *iNKT /10³ T, CD4⁺ iNKTx10⁵/kg CD45RA⁺CD4⁺T/10³ T, CD45RA⁻CD4⁺T/10³ T, CD4⁺ iNKT expansion factor. Data of CD4⁺*
 633 *iNKT/10³ T frequency and CD4⁺ iNKT expansion factor were analyzed as log transformed continuous variables. * Model 2,*
 634 *which analyzes the interactions between stem cell source and the mentioned graft iNKT parameters, was adjusted to donor*
 635 *type and disease status at SCT.*

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644 **Table 4: ROC analyses in all patients and subgroups**

Graft factor	PT N	GVHD events	Cut off	Sensitivity (%)	Specificity (%)	AUC 2.5th percentile	AUC	AUC 97.5th percentile
A. All patients								
CD4⁺ iNKT/10³ T								
Total population	117	29	0.13	92	44.8	60	71.3	82.7
Total BM grafts	33	9	0.14	95.8	44.4	51.5	73.8	96.2
Total PBSC grafts	84	20	0.13	90.7	45	57.2	70.7	84.3
Total population with expansion factor analyzed	96	25	0.13	91.5	52	62	73.8	85.7
CD4⁺ iNKT expansion factor								
Total population with expansion factor analyzed	96	25	6.83	94.4	64	63.6	76.7	89.8
Total BM grafts with expansion factor analyzed	19	7	6.83	91.7	57.1	38.5	67.9	97.2
Total PBSC grafts with expansion factor analyzed	77	18	8.7	94.9	66.7	61.2	77.2	93.2
B. Patients transplanted with HLA matched donor, non progressive disease, expansion factor analyzed								
CD4⁺ iNKT/10³ T								
PBSC and BM grafts	79	17	0.13	91.9	52.9	55.0	69.1	83.2
BM grafts	19	7	0.14	91.7	57.1	51.5	73.8	96.2
PBSC grafts	60	10	0.13	92	50	58	76.2	94.1
CD4⁺ iNKT expansion factor								
PBSC and BM grafts	79	17	6.83	93.5	82.4	80.3	90	99.8
BM grafts	19	7	6.83	91.7	57.1	38.5	67.9	97.2
PBSC grafts	60	10	8.7	94.0	100	96.4	98.6	100

645 *AUC: area under the curve, BM: bone marrow, GVHD events: grade II-IV acute GVHD, N: number, PBSC:*
646 *peripheral blood stem cells, PT: patient*

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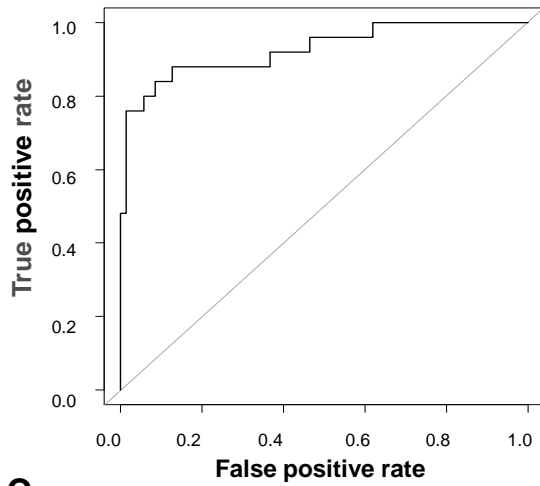
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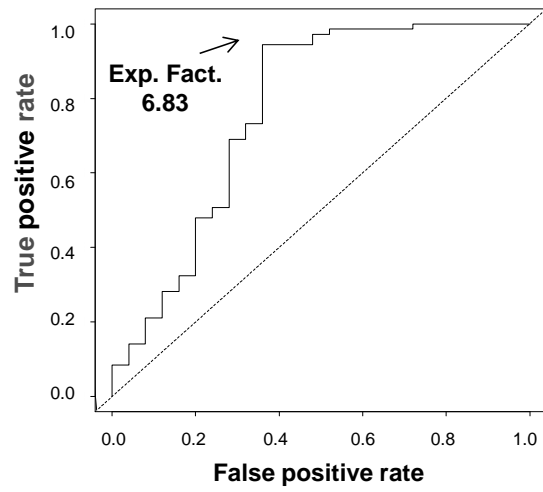
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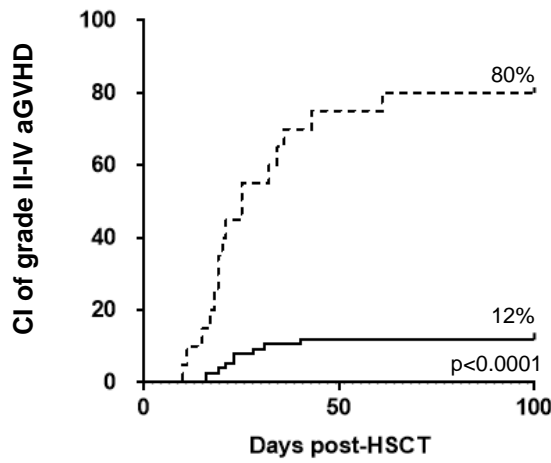
A ROC curve , AUC=0.927



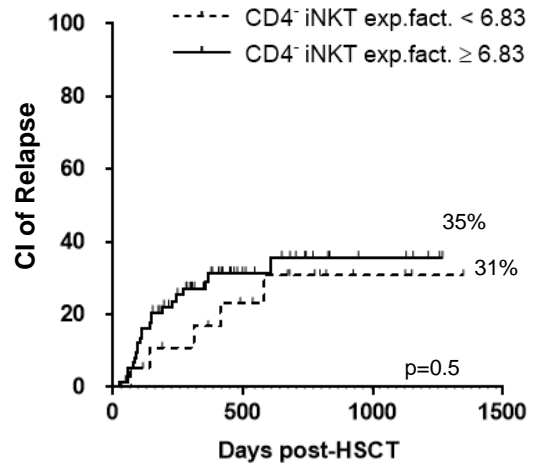
B ROC curve , AUC=0.767



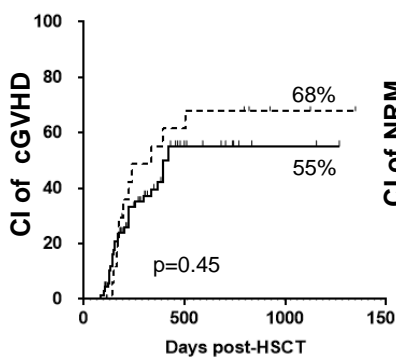
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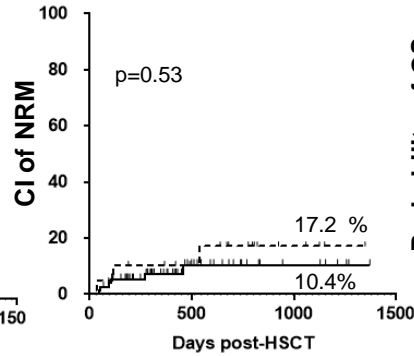
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