Natural killer-cell counts are associated with molecular relapse-free survival after imatinib discontinuation in chronic myeloid leukemia: the IMMUNOSTIM study

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 doi:10.3324/haematol.2017.165001

Received: January 21, 2017.

Accepted: May 8, 2017.

Pre-published: May 18, 2017.

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Online Supplement

Supplementary Methods

Immunophenotyping

To analyze T-cells, NK-cells and their subsets by flow cytometry, a 4-color staining of fresh blood was performed followed by red blood cell lysis. Leucocytes were acquired with a BD Biosciences a FACSCalibur™ and data were analyzed with the BD CellQuest software. PerCP-CD3 (clone SK7), PerCP-CD4 (clone SK3), PE-CD4 (clone SK3), PerCP-CD8 (clone SK1), PE-CD8 (clone SK1), APC-CD45RA (clone HI100), PE-CD27 (clone M-T271), APC-CD56 (clone B159), FITC-CD25 (clone 2A3) and FITC-CD16 (clone 3G8) were from BD Biosciences. FITC-CCR7 (clone 150503) was from R&D Systems). PE-CD127 (clone R34), and PE-NKG2D (clone ON72) were from Beckman Coulter. To analyze NK-receptor expression by flow cytometry on thawed PBMCs, an 8-color staining with mAb was performed, leucocytes were acquired with a BD Biosciences FACSCanto™ II and data were analyzed with the DIVA v6.1.3 software. FITC-DNAM-1 (clone DX11), APC-H7-CD3 (clone SK7) and PercP-Cy5.5-CD56 (clone B159) were from BD Biosciences. PE-KIR2D (clone NKVFS1), PE-NKp30 (clone AF29-4D12), APC-NKp46 (clone 9E2) and Vioblue-CD57 (clone TB03) were from Miltenyi Biotec. PE-Cy7-NKG2A (clone Z199) and APC-CD94 (clone HP-3B1) were from Beckman Coulter. BV510-CD16 (clone 3G8) was from Ozyme.

NK-cell functional assays

To analyze CD107a and CD137 expression by flow cytometry on $CD3^{-}CD56^{+}$ NK-cells in a degranulation assay, leucocytes stained with mAbs were acquired with a BD Biosciences FACSCanto™ II and data were analyzed with the DIVA v6.1.3 software. APC-

CD137 (clone 4B4-1), PE-Cy7-CD56 (clone B159) and APC-H7-CD3 (clone SK7) were from BD Biosciences and BV510-CD16 (clone 3G8) was from Ozyme). For IFN-γ detection by flow cytometry in $CD56^{bright}$ NK-cells after cytokine stimulation, IFN-γ intracellular staining was performed using the AlexaFluor488-labelled B27 clone (BD Biosciences). Leucocytes were acquired with a BD Biosciences FACSCanto™ II and data were analyzed with the DIVA v6.1.3 software.

Supplementary Table S1. Leucocytes, lymphocytes and T-cell subsets at baseline

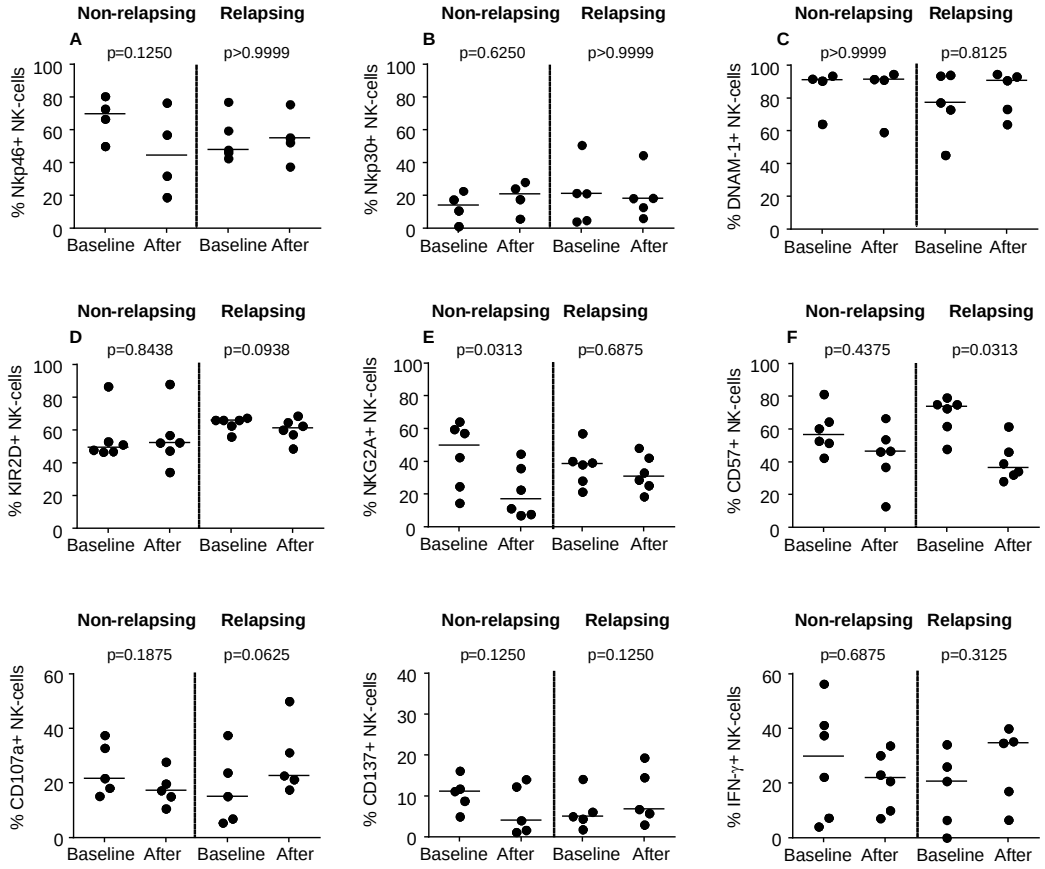
Parameters	All (n=51)	Non-relapsing (n=23)	Relapsing (n=28)	p value*
Leucocytes/mm^3	4820 (2550-7860)	5020 (2550-7470)	4800 (3400-7860)	0.557
Lymphocytes/mm^3	1310 (720-2610)	1400 (810-2610)	1300 (720-1970)	0.185
CD3$^+$ T-cells/mm^3	897 (315-1949)	896 (543-1949)	898 (315-1576)	0.172
CD3$^+$CD4$^+$/mm^3	534 (228-1248)	534 (352-1248)	538 (228-1021)	0.297
CD3$^+$CD8$^+$/mm^3	279 (72-998)	295 (77-998)	276 (72-562)	0.092
Ratio CD4/CD8	2.02 (0.5-14.2)	1.93 (0.5-5.94)	2.09 (0.79-14.2)	0.489
CD3$^+$CD4$^+$ subsets				
Naïve (CD4$^+$CD45RA$^+$CCR7$^+$CD27$^+$)				
% CD4$^+$	35.1 (8.7-75.9)	35.5 (13.1-57.7)	34.7 (8.7-75.9)	0.949
/mm^3	200 (32-774)	189 (82-286)	213 (32-774)	0.949
Central memory (CD4$^+$CD45RA$^-$CCR7$^+$)				
% CD4$^+$	45.1 (19.8-74.7)	46.7 (25.1-74.7)	44.3 (19.8-64.6)	0.339
/mm^3	259 (80-570)	289 (120-570)	223 (80-394)	0.091
Effector memory (CD4$^+$CD45RA$^-$CCR7$^-$)				
% CD4$^+$	15.7 (2.4-53)	13.6 (9.3-29.3)	18.9 (2.4-53)	0.171
/mm^3	91 (25-273)	97 (41-273)	85 (25-232)	0.974
Regulatory (CD4$^+$CD25$^+$CD127$^{low/-}$)				
% CD4$^+$	7 (1.2-13.1)	7.1 (1.2-12.1)	6.4 (3.2-13.1)	0.300
/mm^3	37 (4-94)	40 (4-94)	36 (12-53)	0.136
CD3$^+$ CD8$^+$ subsets				
Naïve (CD8$^+$CD45RA$^+$CCR7$^+$CD27$^+$)				
% CD8$^+$	26.2 (6.5-71.9)	23.7 (6.5-62.7)	28 (7.5-71.9)	0.841
/mm^3	75 (9-239)	75 (28-239)	72 (9-236)	0.342
Central memory (CD8$^+$CD45RA$^-$CCR7$^+$)				
% CD8$^+$	12 (1-38.6)	13.4 (1-23.7)	11.1 (2-38.6)	0.920
/mm^3	30 (2-113)	38 (2-110)	29 (6-113)	0.367
Effector memory (CD8$^+$CD45RA$^-$CCR7$^-$)				
% CD8$^+$	24.2 (1.6-58.3)	20.7 (1.6-58.3)	27.9 (3.2-55.7)	0.325
/mm^3	61 (4-345)	64 (4-345)	56 (9-237)	0.972
Effector memory (CD8$^+$CD45RA$^+$CCR7$^-$)				
% CD8$^+$	33.3 (6.8-84.6)	38.8 (2-110)	33 (6.8-58.7)	0.494
/mm^3	108 (9-416)	111 (20-416)	85 (9-271)	0.211

*The Mann-Whitney U test was used to compare variables from non-relapsing and relapsing patients, with a level of significance of 0.05. Median values (min-max) are shown.

Supplementary Figure S1

Figure S1. NK-cell receptor and function after imatinib discontinuation. (A-F) NK-cell receptor expression, (G) degranulation capacities, (H) activation marker expression and (I) IFN-γ production at baseline and after imatinib discontinuation in non-relapsing (n=6) and relapsing patients (n=6). Scatter dot plots with median are shown. P values (by Wilcoxon matched-pairs signed ranked test) are shown for each set of data.

Figure S1


A
Non-relapsing
Relapsing
p=0.1250
p>0.9999
% Nkp46+ NK-cells
B
p=0.6250
p>0.9999
% Nkp30+ NK-cells
C
p>0.9999
p=0.8125
% DNAM-1+ NK-cells
D
p=0.8438
p=0.0938
% KIR2D+ NK-cells
E
p=0.0313
p=0.6875
% NKG2A+ NK-cells
F
p=0.4375
p=0.0313
% CD57+ NK-cells
p=0.1875
p=0.0625
% CD107a+ NK-cells
p=0.1250
p=0.1250
% CD137+ NK-cells
p=0.6875
p=0.3125
% IFN-γ+ NK-cells
Baseline
After