Table 2. Characteristics of patients with CBF AML

	Auto-SCT			Allo-SCT		
	t(8;21) (n = 61), no.	inv(16) (n = 17), no.	P	t(8;21) (n = 194), no.	inv(16) (n = 66), no.	P
Median age, y (range)	44 (17-68)	37 (19-61)	.59	39 (16-70)	34 (16-64)	.054
Median WBC, g/L (range)	8.8 (0.2-94)	33 (2.1-199)	.02	11 (.6-366)	53 (1.8-284)	< .001
Sex						
Male	41	12	.79	117	40	.93
Female	20	5		74	26	
No. of induction chemotherapy at diagnosis of AML						
1 course	48	15	.72	125	55	.002
> 1 or failure*	11	2		56	7	
Additional cytogenetic abnormalities						
None	53	15	> .999	153	54	.61
Positive	8	2		41	12	
Disease status at SCT						
CR	55	16	> .999	108	52	< .001
Not in CR	6	1		85	11	
CR1	43	13	.98	49	21	.29
CR2	7	1		45	26	
CR3	0	1		5	4	
Conditioning regimen						
TBI	0	1	.22	118	47	.078
Not TBI	61	16		71	16	

Correlation between the two groups was examined.
WBC indicates white blood cell count; g/L, 10^9/L; CR1, first complete remission; and CR2 or 3, second or third CR.
*More than 1 or failure includes patients who did not achieve complete remission after first course of induction chemotherapy, and those who were resistant to induction chemotherapy.

in second or third CR was 45%, 86%, 57%, 44%, and 64%, respectively ($P = .09$). OS of patients undergoing allogeneic SCT not in CR was 18%, 70%, 25%, 15%, and 18%, respectively ($P = .003$).

Table 3. Summary of allogeneic SCT

	t(8;21) (n = 194), no.	inv(16), (n = 66), no.	P
Conditioning regimen			
RIST	31	9	.66
Myeloablative	161	56	
GVHD prophylaxis*			
sMTX+CyA	136	48	.78
sMTX+FK	20	8	
HLA			
Match	146	47	.5
Mismatch	45	18	
Donor			
Related	161	44	.004
Unrelated	32	22	
Stem cell source			
BM	101	40	.27
PB	72	17	
CB	18	7	
aGVHD grade			
0-I	117	37	.54
II-IV	60	22	
cGVHD type			
None	64	28	.28
Lmt/Ext	67	20	

Correlation between the two groups was examined. Some of the missing data was not available, and total numbers do not add up to the number of the patients in each group.

RIST indicates reduced intensity stem cell transplantation; sMTX, short-course methotrexate; CyA, cyclosporin A; FK, tacrolimus; BM, bone marrow; PB, peripheral blood; CB, cord blood; aGVHD, acute graft-versus-host disease; cGVHD, chronic graft-versus-host disease; Lmt, limited; and Ext, extensive.

*Dose of methotrexate was not surveyed in the study. Detail of other GVHD prophylaxis regimens are in Table S1.

When patients undergoing allogeneic SCT in first CR were analyzed, 3-year OS was not significantly different between patients with t(8;21) and inv(16) (84% and 74%, respectively; $P = .28$), between inv(16) and intermediate risk groups (74% and 69%, respectively; $P = .84$), or between t(8;21) and intermediate risk groups (84% and 69%, respectively; $P = .06$). However, when patients undergoing allogeneic SCT in second or third CR were analyzed, the 3-year OS of patients with inv(16) was significantly better than patients with t(8;21) (86% and 45%, respectively; $P = .008$), and better than intermediate risk patients (86% and 57%, respectively; $P = .03$). Difference was not significant between patients in the intermediate risk group and t(8;21) undergoing allogeneic SCT in second or third CR ($P = .36$). The OS of inv(16) patients undergoing allogeneic SCT not in CR was 70% at 3 years, which was also significantly better than that of t(8;21) (18%; $P = .03$) and the intermediate risk group (25%; $P = .045$).

In addition, the OS of t(8;21) undergoing allogeneic SCT in first CR was significantly better than that of the unfavorable risk group (84% and 53%, respectively; $P < .001$), but the difference between the 2 groups was not significant among patients undergoing allogeneic SCT in second or third CR. In contrast, OS was not different between inv(16) and unfavorable groups undergoing allogeneic SCT in first CR, but it was significantly different when they underwent allogeneic SCT in second or third CR (86% and 44%, for inv(16) and unfavorable groups, respectively; $P = .01$) or allogeneic SCT in non-CR (70% and 15%, respectively; $P = .006$).

Survival curves of patients who underwent autologous SCT in first CR, second or third CR, and not in CR are shown in Figure 2A, 2B, and 2C, respectively. The overall survival of patients with t(8;21), inv(16), and intermediate, unfavorable, and unknown cytogenetic risks in first CR was 77%, 59%, 74%, 38%, and 71%, respectively ($P = .049$), while that of patients undergoing autologous SCT in second or third CR was 43%, 50%, 59%, 44%, and 42%, respectively ($P = .8$). The OS of patients undergoing autologous SCT not in CR with t(8;21), inv(16), intermediate, and

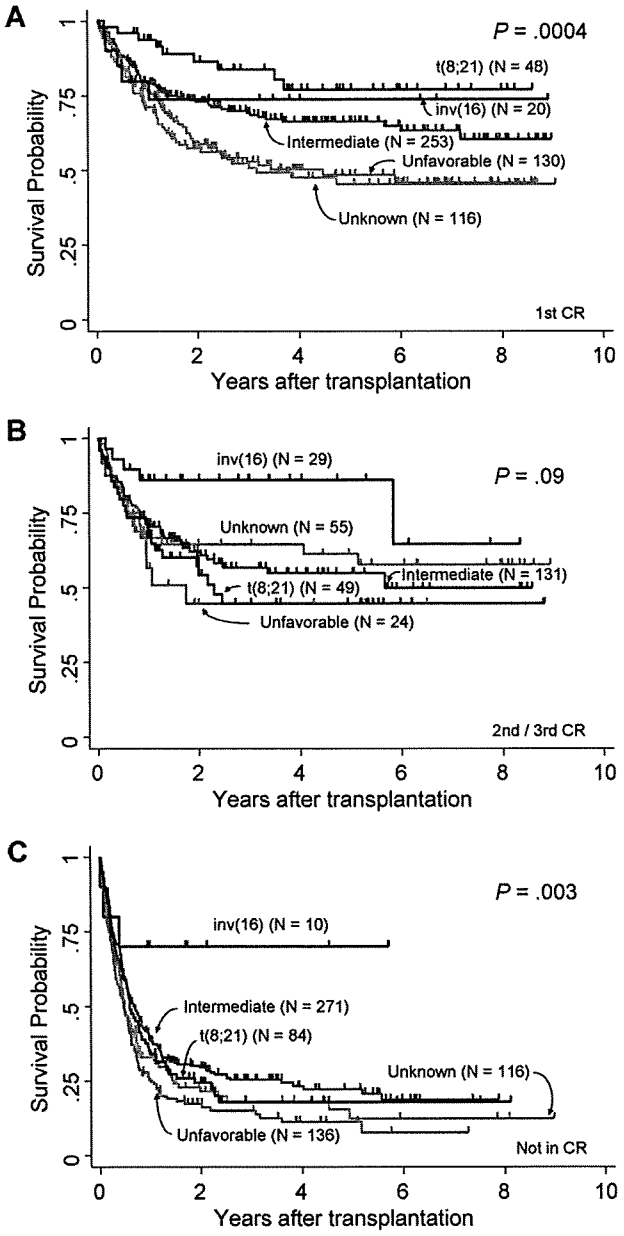

Figure 1. OS difference of patients undergoing allogeneic SCT between cytogenetic subgroups. (A) Survival curves of patients undergoing allogeneic SCT in first CR. (B) Survival curve of patients undergoing allogeneic SCT in second or third CR. (C) Survival curves of patients undergoing allogeneic SCT not in CR. Each are categorized by cytogenetic risk groups, respectively.

unknown risks was 17%, 100%, 25%, and 13%, respectively, and the survival curve of patients in the unfavorable risk group did not reach 3 years ($P = .35$).

Figure 3A and B focus on t(8;21) and inv(16) patients, stratified according to the type of (allogeneic or autologous) and disease status at the time of transplantation (first CR, second or third CR, and not in CR). The 3-year overall survival of t(8;21) patients in first CR was not different between allogeneic and autologous transplantation (84% and 77%, respectively), as well as that of patients in second or third CR (45% and 43%, respectively) and patients not in CR (18% and 17%, respectively). Similarly, the 3-year OS of inv(16) patients was not different between allogeneic and autologous transplantation when they underwent transplantation in first CR (74% and 59%). A significant difference was observed among the 3 disease status groups of t(8;21) patients ($P < .001$; Figure 3A), but not inv(16) patients ($P = .75$; Figure 3B).

The OS of allogeneic SCT, excluding cord blood transplantation, was not different from the analysis presented here, including bone marrow, peripheral blood, and cord blood transplantation (Table S2; Figures S1,S2).

DFS after SCT was also different among cytogenetic risk groups ($P < .001$). DFS of patients with inv(16) (69% at 3 years) was better compared with t(8;21) (49%), intermediate (46%), unfavorable (31%), and unknown (41%) risk groups. Among patients undergoing allogeneic SCT in first CR, DFS was also different among cytogenetic subgroups ($P < .001$). When t(8;21), inv(16), and intermediate cytogenetic subgroups undergoing allogeneic SCT in first CR were compared, the difference was not statistically significant between t(8;21) and inv(16) (78% and 73% at 3 years; $P = .58$), between t(8;21) and intermediate risk group (78% and 63%; $P = .1$), nor between inv(16) and intermediate risk group (73% and 63%; $P = .65$). DFS of patients with t(8;21) undergoing allogeneic SCT in first CR was better than that of the unfavorable risk group (78% and 47%, respectively; $P < .001$), but the difference was not significant between inv(16) and unfavorable risk groups (73% and 47%, respectively; $P = .16$).

DFS was not significantly different when 5 cytogenetic subgroups among patients undergoing allogeneic SCT in second or third CR were compared ($P = .32$). The DFS of patients undergoing allogeneic SCT in second or third CR was not significantly different between t(8;21) and inv(16) (43% and 71% at 3 years; $P = .053$), t(8;21) and the intermediate group (43% and 47%; $P = .76$), or inv(16) and the intermediate group (71% and 47%; $P = .06$). The difference was also not significant between t(8;21) and unfavorable risk groups (43% and 42%; $P = .7$), nor between inv(16) and unfavorable risk groups (71% and 42%; $P = .06$). The DFS of patients undergoing allogeneic SCT who were not in CR was significantly different among the 5 cytogenetic subgroups ($P = .005$), and that of inv(16) (75% at 3 years) was significantly better than t(8;21) (18%; $P = .02$), the intermediate risk group (22%; $P = .03$) and the unfavorable risk group (10%; $P = .003$).

Relapse and TRM

The relapse rate (RR) after SCT also differed among cytogenetic subgroups ($P < .001$). The RR of patients with inv(16) (18% at 3 years) was lower than t(8;21) (38%), intermediate (38%), and unfavorable (56%) risk groups. The RR of t(8;21) and inv(16) after allogeneic SCT was not statistically different in either first CR (16% and 6%; $P = .45$) or second or third CR (34% and 16%, respectively; $P = .09$).

Transplantation-related mortality (TRM) of all patients with AML was 22% at 3 years. The TRM of t(8;21) (18%), inv(16) (11%), and intermediate (21%), unfavorable (24%), and unknown risk groups (27%) was significantly different among cytogenetic risk groups ($P = .02$).

Evaluation of prognostic variables in CBF

Univariate analyses of t(8;21) showed that age ($P = .004$), not in CR at transplantation ($P < .001$), allogeneic SCT ($P = .01$), and TBI regimen ($P = .006$) were significant prognostic factors indicating poor OS (Table 5). Multivariate analysis for OS revealed older age ($P = .01$) and not in CR at transplantation ($P < .001$) as the independent prognostic variables. Univariate analyses of t(8;21) patients who received allogeneic SCT in CR showed that age ($P = .02$), TBI regimen ($P = .01$), and second and third CR at

Table 4. Outcome of the AML patient population by cytogenetic risk groups

	t(8;21)		inv(16)		Intermediate		Unfavorable		Unknown		
	%	N	%	N	%	N	%	N	%	N	*P*
OS											
Allogeneic SCT											
CR1	84	48	74	20	69	253	53	130	52	116	< .001
CR2/CR3	45	49	86	29	57	131	44	24	64	55	.09
Non-CR	18	84	70	10	25	271	15	136	18	116	.003
Autologous SCT											
CR1	77	42	59	13	74	89	38	15	71	39	.05
CR2/CR3	43	7	50	2	59	15	44	6	42	18	.8
Non-CR	17	6	100	1	25	16	0	10	13	8	.35
DFS											
Allogeneic SCT											
CR1	78	48	73	19	63	249	47	129	48	113	< .001
CR2/CR3	43	48	71	27	47	129	42	22	57	54	.32
Non-CR	18	81	75	8	22	255	10	128	16	107	.005
Autologous SCT											
CR1	73	41	62	13	64	81	33	15	61	36	.09
CR2/CR3	43	7	50	2	36	14	50	6	39	18	.89
Non-CR	17	6	100	1	25	16	0	10	17	6	.45

transplantation ($P < .001$) were also significantly prognostic for poor OS. These variables remained significant after multivariate analysis. Univariate analyses for inv(16) patients showed only age ($P = .009$) to be a significant prognostic factor (Table 5). The univariate analysis of inv(16) patients who underwent allogeneic SCT in CR showed only additional karyotype abnormalities to be an unfavorable prognostic variable ($P = .009$).

Additional cytogenetic abnormalities to CBF

A total of 49 patients with t(8;21) and 14 with inv(16) had additional cytogenetic abnormalities. Data for additional cytogenetic abnormalities were obtained in 42 patients with t(8;21) and 13 patients with inv(16) (Table 6). Additional abnormalities were selected that have been reported to be prognostic by others, including loss of sex chromosome (X or Y), trisomy 8, trisomy 4, del(7q), and del(9q) for the t(8;21) group, and trisomy 22, trisomy 8, trisomy 21, del(7q), and del(9q) for the inv(16) group.[14,15,20,22,23] There were no patients with trisomy 21 in the data of patients with CBF. Patients with t(8;21) and patients with inv(16) were analyzed separately. Among t(8;21) patients undergoing allogeneic SCT, survival was not different between patients with and without additional karyotype abnormalities. When patients with inv(16) were analyzed, the survival was not different between patients with (n = 13) and without (n = 67) additional abnormalities (61% and 74%, respectively; $P = .07$). The survival of patients undergoing allogeneic SCT without additional abnormality (n = 52) was significantly better than that with additional abnormality (n = 11), (85% and 53%, respectively; $P = .004$). When analysis was restricted to patients in CR with inv(16) undergoing allogeneic SCT, a similar difference was observed (86% without additional abnormality [n = 42], and 60% with additional abnormality [n = 8], respectively; $P = .03$). Difference in OS was observed among non-CR patients with (n = 9) and without (n = 1) additional abnormality, but this difference may not be relevant with too few patients in the analysis. We further analyzed subgroups of additional abnormalities of the patients with inv(16). Although the number of patients were limited, significant difference was found among 3 groups of patients; trisomy 8 or trisomy 22 as a sole abnormality (n = 4), without additional abnormality (n = 69), and other additional abnormality to inv(16) (n = 10). The OS at 3 years were 100%, 74%, and 42%, respectively ($P = .002$). The OS of patients undergoing allogeneic SCT was also different among these 3 groups (100%, n = 3; 85%, n = 52; and 33%, respectively; $P < .001$).

Discussion

We analyzed the outcome of a large group of patients with adult CBF AML in Japan who were treated with SCT. The current study focused on the different outcome of the 2 different cytogenetic subgroups of patients with CBF AML undergoing SCT. Our study demonstrated a comparable outcome between patients with t(8;21) and inv(16) undergoing SCT in first CR, but the prognosis between these 2 cytogenetic subgroups was different beyond first CR.

In the literature, there have been several reports showing inferior survival of patients with t(8;21) compared with inv(16) patients undergoing induction chemotherapy and SCT.[14,15,20] Other studies categorized both patients with t(8;21) and inv(16) undergoing allogeneic SCT together as good-risk CBF AML,[1,21] with a relatively comparable prognosis. In our study, OS of patients with t(8;21) undergoing allogeneic SCT in first CR was not statistically different from intermediate cytogenetic subgroup (84% and 79% at 3 years, respectively; $P = .058$). Moreover, the survival of inv(16) (74% at 3 years) and intermediate cytogenetic subgroups showed no statistically significant difference.

In contrast, we have here demonstrated that the prognosis of patients with t(8;21) undergoing allogeneic SCT with second or third CR disease was significantly poor compared with those with inv(16). This finding is consistent with those of other studies reporting differences between the 2 types of CBF AML.[14,15] In the present study, non-CR disease with t(8;21) was also significantly poor compared with patients with inv(16). The Acute Leukemia French Association reported that allogeneic donor availability among patients with CBF AML who were in second CR was a prognostic factor for better survival.[16] We believe that different treatment strategies should be applied for patients with t(8;21) and those with inv(16) other than first CR.

Patients with t(8;21) undergoing allogeneic SCT and autologous SCT had a similar survival rate when they underwent transplantation in first CR, and in further CR. No survival difference between allogeneic SCT and autologous SCT was also

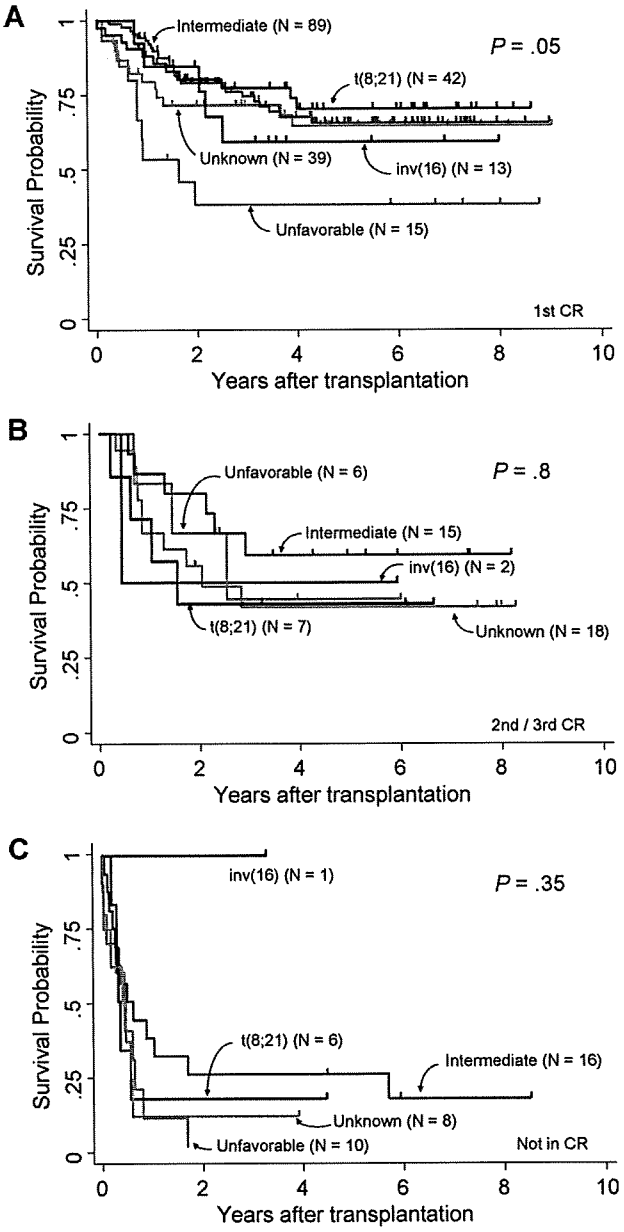

Figure 2. OS difference of patients undergoing autologous SCT between cytogenetic subgroups. (A) Survival curves of patients undergoing autologous SCT in first CR. (B) Survival curves of patients undergoing autologous SCT in second or third CR. (C) Survival curves of patients undergoing autologous SCT not in CR. Each are categorized by cytogenetic risk groups, respectively.

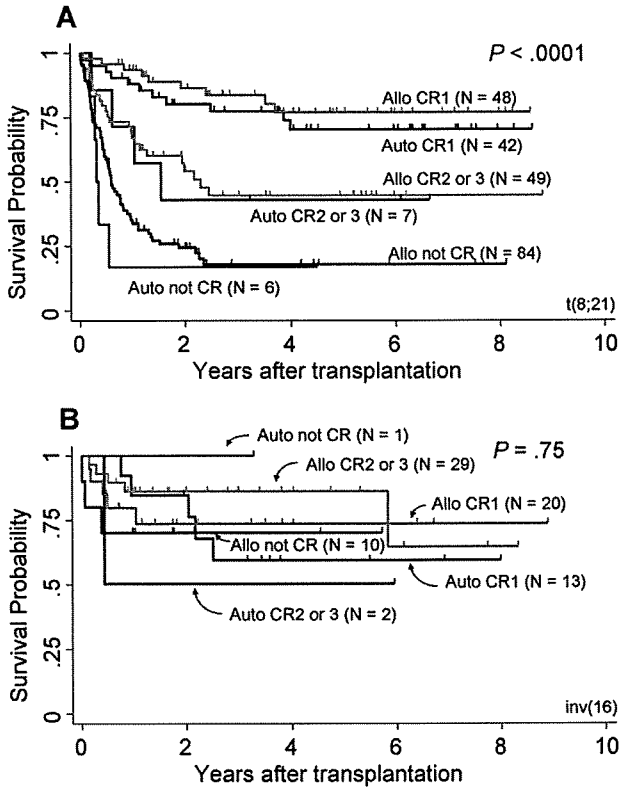

Figure 3. OS of patients with CBF. Survival curves of patients with t(8;21) (A) and with inv(16) (B). Both are stratified according to the type of transplantation (allogeneic or autologous) and disease status at the time of transplantation (first CR, second or third CR, and not in CR).

observed among inv(16) patients receiving SCT in first CR (74% and 59%, respectively). The University of California, San Francisco (UCSF) group described the good results of patients with advanced AML undergoing autologous SCT in second or third remission, including patients with CBF.[24] As in our study, the European Group for Blood and Marrow Transplantation (EBMT) reported that the survival rate of t(8;21) patients who received allogeneic bone marrow transplantation was not significantly different from that of patients who received autologous SCT.[1] Results by others showed that allogeneic SCT in first CR did not benefit good-risk cytogenetic subgroups.[3,25,26] Schlenk et al also demonstrated that t(8;21) patients receiving allogeneic SCT or chemotherapy showed no difference in outcome.[23] These results suggest that autologous SCT can be considered as postremission therapy for patients with CBF AML, but it remains unclear whether SCT is more beneficial for patients with CBF than high-dose cytarabine. Survival of patients with inv(16) was favorable beyond first CR. Patients with inv(16) in second or third CR, or even non-CR patients, are good candidates for allogeneic SCT. There are long-term survivors after allogeneic SCT in non-CR disease, so t(8;21) patients with no other choice of treatment, such as those in further CR or non-CR, can proceed to allogeneic SCT. In order to confirm the appropriate treatment for t(8;21) patients in first CR, a prospective trial is needed to compare the results of autologous SCT for t(8;21) in first CR with standard chemotherapy. t(8,21) patients with suitable related or well-matched donors should be recommended to participate in a risk-adopted prospective trial when they receive allogeneic SCT in first CR.

There were differences between the 2 types of CBF AML with respect to prognostic valuables. Age was a significant and independent prognostic variable in both t(8;21) and inv(16) patients, a finding in agreement with reports from some,[14,27] but not all,

Table 5. Prognostic factors affecting overall survival of patients with t(8;21)

Variables	Unfavorable factors	Hazard ratio	95% CI	P
t(8;21)				
Age		1.02	1.01-1.04	.004
Disease status at SCT	Not in CR	4.4	3.1-6.5	< .001
Transplantation	Allo-SCT	1.9	1.2-3.0	.01
Conditioning regimen	TBI	1.7	1.2-2.5	.005
inv(16)				
Age		1.1	1.0-1.1	.009

CI indicates confidence interval.

Table 6. Additional cytogenetic abnormalities among patients with CBF

Additional cytogenetic abnormalities	t(8;21), no.	inv(16), no.
None	206	69
With additional abnormalities	49	14*
−Y	10	0
−X	5	0
Trisomy 22	0	3†
Trisomy 8	0	2†
Trisomy 4	2*	0
Complex	7	4
del(7q)	1†	2
del(9q)	6	0
Other abnormalities	27	9‡
Unknown	7	1

*Patients with additional change to inv(16) and trisomy 4 with t(8;21) tended to show poor survival tendency, with $P < .1$.

†All patients with trisomy 22, trisomy 8 with inv(16), and del(7q) with t(8;21) were alive and censored at survival analysis.

‡Other abnormalities with inv(16) was poorly prognostic, with $P < .001$.

investigators.[28] Transplantation in CR was a significant and independent prognostic factor for patients with t(8;21), but not for those with inv(16). The Cancer and Leukemia Group B (CALGB) also reported differences between t(8;21) and inv(16) in prognostic factors, in terms of race, sex, and secondary cytogenetic abnormalities.[14] Among patients with CBF AML, t(8;21) and inv(16) patients undergoing SCT should be considered 2 separate clinical entities in future clinical studies.

Several specific additional karyotype abnormalities have been reported to be prognostic in patients with CBF AML. Among t(8;21) patients, no specific additional karyotype abnormality was prognostic for overall survival. The poor prognosis of t(8;21) patients with trisomy 4 has been reported by others,[22] but the survival difference was not statistically significant ($P = .085$) in our case series. Since there were limited numbers of patients with additional abnormalities, the real significance of each additional abnormality should be investigated in large numbers of patients.

The reason for the different survival results between patients with t(8;21) and inv(16) undergoing allogeneic SCT in our study remains unclear. The impact of additional mutational events such as c-Kit, FLT3, RAS, and gene-expression profiles was reported to be associated with the clinical outcome of patients with CBF AML.[29-34] The effects of these additional mutational events and gene-expression profiles on the clinical outcome of autologous and allogeneic SCT have not yet been studied. Which proportion of the patients with CBF AML benefited from earlier SCT remains to be identified in future clinical studies. Recent studies by others also suggested that prognosis of CBF AML could differ among different ethnic groups or races.[14,35-37] The background molecular basis among the Japanese population must also be taken into account in future studies.

In conclusion, the survival outcome of patients with CBF AML was similar when they received allogeneic or autologous SCT in first CR. However, the outcomes were significantly different between t(8;21) and inv(16) when they received allogeneic SCT beyond first CR. Therefore, these 2 kinds of CBF AML should be managed differently when applying SCT.

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Authorship

Contribution: Y. Kuwatsuka, K.M., and R.S. contributed to data collection, designed and performed the study, analyzed the data, and wrote the manuscript; M.K., A.M., H.O., R.T., S.T., K.K., K.Y., Y.A., T.Y., and H.S. contributed to data collection and analysis and writing of the paper; and Y. Kodera contributed to data collection and writing of the paper, conceived the study, and provided intellectual input.

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Impact of macrophage infiltration of skin lesions on survival after allogeneic stem cell transplantation: a clue to refractory graft-versus-host disease

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Brief report

Impact of macrophage infiltration of skin lesions on survival after allogeneic stem cell transplantation: a clue to refractory graft-versus-host disease

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We retrospectively reviewed 104 biopsy specimens of previously untreated skin acute graft-versus-host disease (GVHD) within 100 days after allogeneic stem cell transplantation, and analyzed the relationship between types of infiltrating cells and clinical outcomes. Counting the total number of CD8$^+$ T cells, CD163$^+$ macrophages, and CD1a$^+$ dendritic cells in 4 fields under original magnification ×200, the infiltration of more than 200 cells of CD163$^+$ macrophages (many macrophages [MM]) was the only significant predictor for refractory GHVD (odds ratio, 3.79; 95% confidence interval, 1.22-11.8; $P = .02$). In 46 patients given steroid treatments, MM was the only significant predictor for refractory acute GVHD (odds ratio, 5.05; 95% confidence interval, 1.19-21.3; $P = .03$). Overall survival of patients with MM was significantly lower than that of those with an infiltration of less than 200 cells of CD163$^+$ macrophages. Macrophage infiltration of skin lesions could be a significant predictive factor for refractory GVHD and a poor prognosis. (Blood. 2009;114: 3113-3116)

Introduction

Macrophages are phagocytic cells with various abilities, such as phagocytosis, antigen-presenting, and secretion of cytokines.[1,2] Recently, it was revealed in human sequential biopsy data that recipient macrophages contributed to acute graft-versus-host disease (GVHD) by antigen-presenting and secreting cytokines, causing the activation and proliferation of CD8$^+$ T cells.[3] We focused on macrophage involvement in acute GVHD, especially on the relationship between the macrophage infiltration of skin lesions and refractory GVHD.

Methods

Between January 1997 and October 2007 at the Japanese Red Cross Nagoya First Hospital, we used skin biopsy specimens within 100 days after allogeneic stem cell transplantation (allo-SCT) of skin lesions clinically considered acute GVHD without any GVHD treatment from 104 patients who underwent allo-SCTs. We analyzed the relationship between types of infiltrating cells and clinical outcomes by counting the total number of CD8$^+$ T cells, CD163$^+$ macrophages, and CD1a$^+$ dendritic cells in 4 fields of a skin biopsy specimen under original magnification ×200. Immunohistochemical analysis using paraffin sections was performed using monoclonal antibodies against CD8, CD163, and CD1a (Novocastra). CD163 is a member of the scavenger receptor cystein-rich superfamily and is an exclusive marker for macrophages, playing a major role in the scavenging components of damaged cells.[4-7]

The endpoints of this study were the outcomes of acute GVHD and overall survival (OS). Acute GVHD was diagnosed and graded according to the consensus criteria.[8] We defined refractory GVHD as that exhibited by patients who had persistent lesions after primary steroid treatments. To establish parameters, we analyzed the numbers of infiltrating CD8$^+$ T cells ($\leq$ 100/4 fields [few T cells; FT] vs $>$ 100/4 fields [many T cells; MT]), numbers of infiltrating CD163$^+$ macrophages ($\leq$ 200/4 fields [few macrophages; FM] vs $>$ 200/4 fields [many macrophages; MM]), disease risk (low vs high), human leukocyte antigen (HLA) disparity (match vs mismatch), donor source (related vs unrelated), graft source (bone marrow vs peripheral blood), age at allo-SCT ($\leq$ 50 years vs $>$ 50 years), conditioning regimen (conventional regimens vs reduced intensity regimens), and skin GVHD stage at biopsy (stages 1-2 vs stages 3-4). A significance level of $P < .05$ was used for all analyses, which were based on all data available as of August 31, 2008. Protocols were approved by the Japanese Red Cross Nagoya First Hospital's Institutional Review Board, and all patients provided informed consent in accordance with the Declaration of Helsinki.

Results and discussion

Table 1 summarizes the characteristics of patients and information gathered about GVHD. We divided patients into 4 groups according to the amount of infiltrating cells (FM and FT, 60.6%; MT and FM, 18.2%; MT and MM, 10.6%; and FT and MM, 10.6%). We noted a striking difference among patients in the types of infiltrating cells in skin GVHD lesions (Figure 1A). The distributions of numbers of infiltrating cells also exhibited a

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Table 1. Information on patient characteristics, acute GVHD, and skin biopsy

Characteristic	Value
Patient characteristics	
Total no. of patients	104
Median age at allo-SCT, y (range)	40.5 (19-61)
Male/female	65/39
Disease risk, low/high	51/53
HLA, match/mismatch	72/32
Donor, unrelated/related	67/37
Graft, BM/PB/CB	89/11/4
Conditioning, conventional/RIST	78/26
Median observation period, mo (range)	13.7 (0.7-120.7)
Acute GVHD	
Stage skin (at the time of biopsy), 1/2/3/4	22/57/25/0
Skin (the maximal severity), 1/2/3/4	16/25/52/11
Gut, 0/1/2/3/4	69/9/8/15/3
Liver, 0/1/2/3/4	82/5/3/9/5
Grade (at the time of the biopsy), I/II/III/IV	58/41/4/1
Grade (the maximal severity), I/II/III/IV	28/44/19/13
Primary steroid treatment, yes/no	46/58
Second treatment, yes/no	18/30
Outcome of GVHD, improved/refractory	84/20
Skin lesion	
Median date of appearance, days (range)	24 (5-81)
Median date of skin biopsy, days (range)	31.5 (6-82)
Median date of highest stage of skin GVHD, days (range)	34 (9-90)
No. of infiltrating $CD8^+$ cells	65 (2-305)
No. of infiltrating $CD163^+$ cells	132.5 (38-372)
No. of infiltrating $CD1a^+$ cells	7 (0-122)

Disease risk low indicates acute leukemia in first remission; CML, in first chronic phase; MDS, refractory anemia or nonmalignant hematologic disease; disease risk high, all other diagnoses; HLA match, identical HLA-A, -B, and -DRB1 loci; HLA mismatch, at least one disparity at one of these loci; BM, bone marrow; PB, peripheral blood; CB, cord blood; and RIST, reduced intensity conditioning regimens.

considerably wide variety (Figure 1B). The median number of infiltrating $CD8^+$ T cells was 65 (range, 2-305), that of infiltrating $CD163^+$ macrophages was 132.5 (range, 38-372), and thatof infiltrating $CD1a^+$ dendritic cells was 7 (range, 0-122). We used 3 skin biopsy specimens of drug rash from autologous transplantation patients as non-GVHD controls; the median numbers of $CD8^+$, $CD163^+$, and $CD1a^+$ infiltrating cells were 11 (range, 6-15), 26 (range, 19-30), and 68 (range, 65-83), respectively. MT was correlated with an HLA mismatch ($P = .047$), grade III-IV acute GVHD ($P = .03$), and MM ($P = .01$), whereas MM was correlated with unrelated donor ($P = .04$), an HLA mismatch ($P = .049$), refractory GVHD ($P = .004$), and MT ($P = .01$) using χ^2 analyses. The sensitivity and specificity of MT for refractory GVHD were 25.0% and 70.5% in all 104 patients, and 25.0% and 73.3% in 46 receiving steroids, whereas those of MM were 43.8% and 82.9%, and 43.8% and 86.7%, respectively.

In 46 patients undergoing steroid treatments, the median date of the appearance of skin lesions was 17.0 days (range, 5-54 days), whereas that of skin biopsy was 27.5 days (range, 6-63 days) and that of the highest skin stage was 32.0 days (range, 9-68 days). Treatments for GVHD were considered for GVHD patients without spontaneous regression and with progression to a higher grade, except for those in which enhanced immunosuppression would not be preferable, such as encephalopathy resulting from calcineurin inhibitor or a pathologic diagnosis of intestinal transplantation-associated microangiopathy.[9,10] The median interval from the initial clinical manifestation of GVHD to the primary treatments was 4.5 days (range, 0-97 days). The dose of prednisolone was 0.5 mg/kg in 4 patients, 1 mg/kg in 20 patients, 2 mg/kg in 19 patients, 500 mg/body in 1 patient, and 1000 mg/body in 2 patients. Only MM was identified as a negative predictive factor for refractory GVHD (Table 2). In 46 patients undergoing steroid treatments, only MM was identified as a negative predictive factor for refractory GVHD (odds ratio, 5.05; 95% confidence interval [CI], 1.19-21.3; $P = .03$).

In the Cox proportional hazard model, age more than 50 years, high risk, and MM were identified as significant risk factors by univariate analyses, with MM and high risk remaining a significant risk in a multivariate analysis (Table 3). OS rates were significantly higher in FM patients compared with those in MM (Figure 1C). Uncontrolled GVHD was the cause of 6 MM patients of 11 (54.5%) who died because of transplantation-related mortality (TRM), whereas in FM patients, 3 of 24 (12.5%) died because of uncontrolled GVHD. The causes of death for the 5 MM patients who did not die of uncontrolled GVHD were infection in 3 patients, intestinal transplantation-associated microangiopathy in 1 patient, and liver failure in 1 patient. In 46 patients who underwent steroid treatments, only MM was identified as a significant risk factor in the Cox proportional hazards model (Hazard ratio 3.25; 95% CI, 1.46-7.26; $P = .004$). OS rates were significantly higher in FM patients compared with those in MM (Figure 1D). Uncontrolled GVHD was the cause of 6 MM patients of 9 (66.7%) who died because of TRM, whereas in FM patients, 3 of 15 (20.0%) died because of uncontrolled GVHD.

Our study suggested that macrophages are involved in a specific type of acute GVHD that tended to be systemic and refractory to conventional therapies, such as corticosteroids or calcineurin inhibitors.[11-13] Differences in treatment efficacy could be explained by the difference in infiltrating cell types. Although efforts have been made at predicting refractory GVHD,[14-17] no confirmed factor has been established to date. Our findings could prove to be a relatively simple and useful method directly related to the prognosis of patients.

Macrophages could not be completely suppressed by current therapies for acute GVHD mainly targeting T cells. Considered together with

Table 2. Analyses of predictive factors for refractory GVHD in all 104 patients

Parameter	Odds ratio (95% CI)	P
More than 50 y old	1.21 (0.35-4.19)	.76
High disease risk	1.29 (0.44-3.76)	.65
Graft PB (vs BM)	1.19 (0.23-6.11)	.83
Unrelated donor	0.91 (0.30-2.73)	.86
HLA mismatch	1.96 (0.66-5.84)	.23
Conventional regimens	0.94 (0.27-3.23)	.92
Skin stage 3 or 4 at biopsy	1.97 (0.69-5.68)	.21
MT (> 100 $CD8^+$ cells)	1.26 (0.37-4.27)	.71
MM (> 200 $CD163^+$ cells)	3.79 (1.22-11.8)	.02

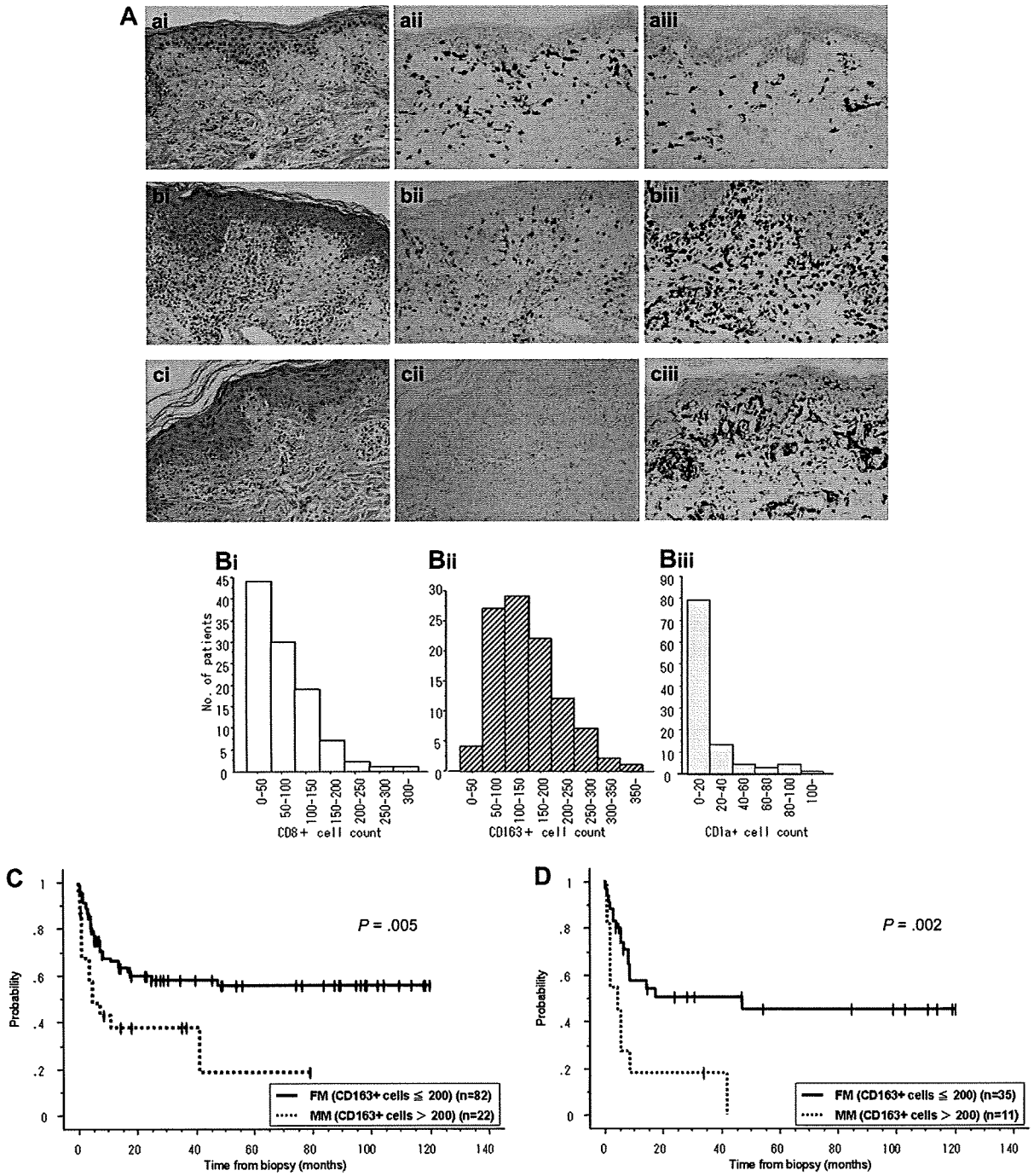

Figure 1. Skin biopsy specimens, infiltrating cells, and overall survival. Immunohistochemical analysis of representative skin biopsy specimen (A), the cell count distribution of $CD8^+$, $CD163^+$, and $CD1a^+$ cells (B), and the impact of MM on OS (C-D). (A) Tissue sections of skin biopsy were stained with hematoxylin and eosin (ai,bi,ci), or antibodies to $CD8^+$ (aii, bii, cii), or $CD163^+$ (aiii, biii, ciii) as detailed in "Methods." Shown are representative specimens of an MT/FM patient (ai-iii), a MT/MM patient (bi-iii), and an FT/MM patient (ci-iii). Original magnifications ×200. (B) Distribution of infiltrating cell counts (Bi, $CD8^+$; Bii, CD163; and Biii, CD1a). (C) OS according to $CD163^+$ cell counts (≤ 200 [FM] vs > 200 [MM]) in all patients. OS of patients with MM was significantly lower than that of those with FM (FM: 66.2% ± 10.6% at 1 year and 58.3% ± 11.4% at 3 years, MM: 37.8% ± 21.0% at 1 year and 37.8% ± 21.0% at 3 years, respectively; $P = .005$). (D) OS according to $CD163^+$ cell counts (≤ 200 vs > 200) in 46 patients undergoing steroid treatments. OS of patients with MM was significantly lower than that of those with FM (FM: 57.7% ± 17.1% at 1 year and 50.7% ± 17.4% at 3 years; MM: 18.2% ± 22.7% at 1 year and 18.2% ± 22.7% at 3 years, respectively; $P = .002$).

the fact that skin biopsies can be performed safely without any critical complications, our results support the importance of conducting skin biopsies of posttransplantation skin lesions. In cases where macrophage-dominant infiltration is observed in a skin biopsy specimen, macrophage-targeted therapies[18-23] could provide a clue to refractory GVHD.

In conclusion, macrophage infiltration of skin lesions after allo-SCT was shown to be a significant predictive factor for

Table 3. Analyses of risk factors for OS in all 104 patients

	Univariate		Multivariate	
Parameter	Hazard ratio (95% CI)	P	Hazard ratio (95% CI)	P
Older than 50 y	2.27 (1.23-4.20)	.009	—	—
High disease risk	1.87 (1.03-3.40)	.04	1.92 (1.06-3.50)	.03
Graft PB (vs BM)	2.04 (0.91-4.61)	.08	—	—
Unrelated donor	0.71 (0.40-1.29)	.26	—	—
HLA mismatch	1.13 (0.61-2.10)	.70	—	—
Conventional regimens	0.74 (0.39-1.39)	.35	—	—
Skin stage 3 or 4 at biopsy	1.11 (0.57-2.15)	.76	—	—
MT (> 100 $CD8^+$ cells)	1.10 (0.59-2.08)	.76	—	—
MM (> 200 $CD163^+$ cells)	2.38 (1.27-4.49)	.006	2.45 (1.30-4.61)	.006

— indicates not applicable.

refractory GVHD, as well as being a negative prognostic factor for OS. Our results indicate the importance of skin biopsies after allo-SCT and suggest the possibility of developing infiltrating cell-based strategies.

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Authorship

Contribution: S.N., S.T., M.I., and K.M. designed the research; S.N., S.T., and M.I. analyzed and interpreted the data; S.N. and S.T. performed statistical analysis; and S.N., T.G., A.S., K.W., M.Y., N.I., S.T., M.S., Y.O., M.I., and K.M. collected clinical data and wrote the paper.

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The Journal of Immunology

Notch Activation Induces the Generation of Functional NK Cells from Human Cord Blood CD34-Positive Cells Devoid of IL-15

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Notch Activation Induces the Generation of Functional NK Cells from Human Cord Blood CD34-Positive Cells Devoid of IL-15[1]

Kyoko Haraguchi,*† Takahiro Suzuki,[2]*‡ Noriko Koyama,‖ Keiki Kumano,*‡ Fumio Nakahara,[3]*‡ Akihiko Matsumoto,[4]* Yasuhisa Yokoyama,[5]*‡ Mamiko Sakata-Yanagimoto,[5]*‡ Shigeo Masuda,[6]*‡ Tsuyoshi Takahashi,‡ Aki Kamijo,[7]§ Koki Takahashi,§ Minoko Takanashi,# Yoshiki Okuyama,† Koji Yasutomo,** Seiji Sakano,†† Hideo Yagita,‖ Mineo Kurokawa,‡ Seishi Ogawa,‡¶ and Shigeru Chiba[8]*‡‡

The development of NK cells from hematopoietic stem cells is thought to be dependent on IL-15. In this study, we demonstrate that stimulation of human cord blood CD34$^+$ cells by a Notch ligand, Delta4, along with IL-7, stem cell factor, and Fms-like tyrosine kinase 3 ligand, but no IL-15, in a stroma-free culture induced the generation of cells with characteristics of functional NK cells, including CD56 and CD161 Ag expression, IFN-γ secretion, and cytotoxic activity against K562 and Jurkat cells. Addition of γ-secretase inhibitor and anti-human Notch1 Ab to the culture medium almost completely blocked NK cell emergence. Addition of anti-human IL-15-neutralizing Ab did not affect NK cell development in these culture conditions. The presence of IL-15, however, augmented cytotoxicity and was required for a more mature NK cell phenotype. CD56$^+$ cells generated by culture with IL-15, but without Notch stimulation, were negative for CD7 and cytoplasmic CD3, whereas CD56$^+$ cells generated by culture with both Delta4 and IL-15 were CD7$^+$ and cytoplasmic CD3$^+$ from the beginning and therefore more similar to in vivo human NK cell progenitors. Together, these results suggest that Notch signaling is important for the physiologic development of NK cells at differentiation stages beyond those previously postulated. *The Journal of Immunology,* **2009, 182: 6168–6178.**

Natural killer cells are critical for host immunity because they rapidly mediate cellular cytotoxicity against pathogen-infected or malignantly transformed cells and produce a wide variety of cytokines and chemokines that influence other components of the immune system. Unlike other lymphocytic lineages, however, the continuous staging scheme of human NK cell development in vivo has yet to be elucidated (1). One reason for this may be the difficulty in closely correlating our knowledge of mouse NK cell biology with human NK cell biology (2), because mouse NK cells do not express a homolog of CD56, which is the marker most representative of human NK cells; instead, the most widely used markers of NK cells in various mouse strains are NK1.1 and DX5, mouse-specific Ags. Among the molecules involved in NK cell development, IL-15 has a particularly important role. For example, IL-15-deficient mice lack NK1.1$^+$ cells (3), indicating that IL-15 is essential for NK cell development in mice. The requirement of IL-15 for mouse NK cell development has also been demonstrated by other studies (4, 5). In humans, IL-15 is considered to be required for in vitro NK cell development and virtually most current protocols for human NK cell differentiation culture depend on IL-15. IL-15-independent NK cell differentiation has been reported in which human cord blood (CB)[9] cells are cocultured with murine stromal cell lines (6). Signaling, however, substituting IL-15 signaling that is responsible for the NK cell differentiation in this culture system was not described.

NK cells are thought to be derived from hematopoietic stem cells through a T/NK precursor stage. The Notch signaling pathway influences cell fate decisions in numerous cellular systems,

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[9] Abbreviations used in this paper: CB, cord blood; cy, cytoplasmic; FL, Fms-like kinase 3 ligand; DAPT, *N*-[*N*-(3,5-difluorophenacetyl-L-alanyl)]-*S*-phenylglycine *tert*-butyl ester; CMA, concanamycin A.

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including various hematopoietic and immune cells (7–9). To date, four Notch receptors (Notch1– Notch4) and at least four Notch ligands (Delta1, Delta4, Jagged1, and Jagged2) have been identified in mammals. Signaling through Notch1 is crucial in the early stages of T cell development (10–12). In culture, ligand-induced Notch signaling drives human CB $CD34^+$ cells to differentiate into T/NK cell precursors (13). Furthermore, Notch signaling drives the T/NK precursors toward differentiation into T and NK cells, although the results for the NK cells are controversial. For example, inhibition of Notch signaling suppresses T cell development and stimulates NK cell development (14–16), whereas activation of Notch signaling contributes to the efficient development of NK cells in mice (17, 18) and humans (19). It is not concluded, however, whether Notch signaling is involved in the function of NK cells or whether IL-15 is necessary for NK cell development in culture.

In this report, to gain further insight into the physiologic significance of Notch signaling in NK cell development, we examined whether IL-15 is dispensable for the generation of functional NK cells and whether Notch signaling has a role in the later stages of NK cell development. Our results indicated that Notch signaling, but not IL-15 stimulation, was essential for inducing $CD34^+$ cells to give rise to $CD7^+$ and cytoplasmic (cy) $CD3^+$ cells that express CD56 in stroma-free culture. Surprisingly, cells cultured with Delta4-coated plates, but lacking IL-15 in the medium, were functional NK cells with cytotoxic activity. IL-15, along with Delta4, further augmented NK cell activity and phenotypic maturation. The addition of IL-15 without exogenous Notch ligand, however, did not allow $CD34^+$ cells to take a NK cell developmental pathway resembling physiologic NK cell precursors. Notch signaling might have a significant role in the development of NK cells in vivo.

Materials and Methods

Reagents and Abs

Recombinant human Delta4-Fc chimeric protein was generated as described previously (20). Recombinant human IL-7 and IL-15 were purchased from R&D Systems. Human stem cell factor and human Fms-like kinase 3 ligand (FL) were a gift from Amgen. Human IL-6/IL-6 receptor fusion protein (FP6) and human thrombopoietin were provided by Kirin Pharma. Anti-IL-15 Ab (MAB2471) and isotype control mouse IgG1 were purchased from R&D Systems. Anti-CD3 (UCHT1), CD8 (SK1), CD14 (M5E2), CD44 (G44-26), CD45 (HI30), CD45RA (HI100), CD56 (B159), CD94 (HP-3D9), CD161 (DX12), NKG2D (1D11), CCR7 (3D12), granzyme B (GB11), and IFN-γ (25723.1) Abs were purchased from BD Biosciences. Anti-CD2 (T11), CD4 (13B8.2), CD7 (8H8.1), CD11a (25.3), CD11b (Bear1), CD25 (B1.49.9), CD27 (1A4CD27), CD33 (D3HL60.251), CD57 (NC1), CD62L (DREG56), CD117 (YB5.B8), CD122 (CF1), CD158a (EB6), and CD158b (GL183) Abs were purchased from Beckman Coulter. Anti-CD34 and CD133 Abs were purchased form Miltenyi Biotec. RIK-2, anti-TRAIL mAb, was prepared as described previously (21).

Isolation of $CD34^+$ and $CD133^+$ cells

Human CB samples were collected from normal full-term deliveries. The parents of all donors provided written informed consent to participate in the study. The procedures were approved by the institutional review board. Mononuclear cells were separated from blood samples by density gradient centrifugation (Lymphoprep; AXIS-SHIELD PoC). CD34- and CD133-enriched cells were separated from mononuclear cells using a MACS Direct CD34 Progenitor Cell Isolation Kit and MACS CD133 MicroBead Kit (Miltenyi Biotec), respectively, according to the manufacturer's protocol. The purity of the $CD34^+$ and $CD133^+$ cells was 97.3 ± 2.3% (n = 15) and 95.4 ± 3.2% (n = 4), respectively. Residual $CD3^+$ and $CD56^+$ cells were 0.73 ± 0.42% and 0.41 ± 0.32%, respectively, in either purification strategy.

Cell culture

Nontissue culture-type 24-well plates were precoated by applying 10 μg/ml Delta4-Fc or control Fc fragments of human Ig G (Fc) (Athens Research & Technology) to the plates at 37°C for 1 h. Cells were cultured in MEM Eagle, α modification (Sigma-Aldrich) supplemented with 20% FBS (Thermo Trace) and penicillin-streptomycin at 37°C in a humidified atmosphere flushed with 5% CO_2 in air. The number of CD34 or CD133 magnetic bead-sorted cells seeded in each well was 0.25–1.2 × 10^5. Cytokines were added at concentrations of 10 ng/ml for IL-7, 100 ng/ml for stem cell factor and 100 ng/ml for FL. one-half of the culture medium was changed every 3 or 4 days. Ten nanograms of thrombopoietin per ml and 100 ng/ml FP6 were added only into the starting culture medium for effective proliferation, although they were not essential (data not shown). IL-15 was added at 5 ng/ml when indicated. Anti-IL-15 or isotype IgG was added at 10 μg/ml when indicated. To inhibit Notch signaling, 10 μmol/L γ-secretase inhibitor *N*-[*N*-(3,5-difluorophenacetyl-L-alanyl)]-*S*-phenylglycine *tert*-butyl ester (DAPT; Calbiochem) was added to the culture medium. $CD161^+$ and $CD161^-$ cells from the culture were isolated using FACSAria (BD Biosciences) after staining with anti-CD161-PE Ab.

Phenotyping assay

Immunofluorescence staining for flow cytometry was performed according to standard procedures. To exclude dead cells from the analysis, 7-aminoactinomycin D (Beckman Coulter) was used. Cytoplasmic staining was performed as follows: after staining the cells with anti-CD56-allophycocyanin and fixing with FACS lysing solution (BD Biosciences), the cells were permeabilized using FACS permeabilizing solution (BD Biosciences) and stained with anti-CD3-PE Ab. For staining for granzyme B, the same fixing and permeabilizing procedure was performed after cell surface staining with anti-CD56-PE and anti-CD3-allophycocyanin. For staining for TRAIL, the cells were incubated with 1 μg of RIK-2 for 30 min at 4°C followed by anti-mouse IgG1-PE (A85-1). Cells were analyzed by flow cytometry using FACSCalibur and CellQuest software (BD Biosciences).

Cytotoxicity assays

A ^{51}Cr release assay to determine cytotoxicity was performed using standard procedures. In brief, 5 × 10^3 K562 or Jurkat cells were labeled with $Na_2{}^{51}CrO_3$ (Amersham Biosciences) and cocultured with effector cells at various ratios in 96-well round-bottom microtiter plates in 200 μl of culture medium. The cocultured cells were incubated for 4 h, and 100 μl of supernatant was collected from each well and counted with a Packard COBRA gamma counter (Packard Instruments). The percentage of specific ^{51}Cr release was calculated as follows: [cpm experimental release − cpm spontaneous release]/(cpm maximal release − cpm spontaneous release) × 100. The ratio of spontaneous release to maximal release was <20% in all experiments. In experiments to test the mode of cytotoxicity, we used concanamycin A (CMA; Sigma-Aldrich) as a selective inhibitor of the perforin-mediated cytotoxicity, and anti-TRAIL Ab RIK-2. Effectors were pretreated with 100 nmol/L CMA for 2 h before the cytotoxicity assays (22). RIK-2 was added at a final concentration of 10 μg/ml at the start of the cytotoxicity assay.

Intracellular cytokines

The cells were stimulated by PMA (25 ng/ml; Sigma-Aldrich) and ionomycin (1 μg/ml; Sigma-Aldrich) in the presence of monensin (2 μmol/L; Sigma-Aldrich) for 4 h. After staining the cells with anti-CD56-PE, they were fixed and permeabilized as described above and stained with anti-IFN-γ-FITC Ab. The cells were analyzed on a FACSCalibur using CellQuest software.

Anti-Notch Abs

For cell surface staining, we used biotinylated Abs and streptavidin-PE (BD Biosciences). To block Notch1, we added 10 (μg/ml) MHN1-519 to the medium. Mouse IgG1 (R&D Systems) was used as the control. The anti-human Notch1 (MHN1-519, mouse IgG1), Notch2 (MHN2-25, mouse IgG2a), and Notch3 (MHN3–21, mouse IgG1) mAbs were generated by immunizing BALB/c mice with human Notch1-Fc (R&D Systems), Notch2-Fc (the Fc portion of human IgG1 was fused to the 22nd epidermal growth factor repeat of the extracellular region of human Notch2), or Notch3-Fc (R&D Systems) and screening hybridomas producing mAbs specific for Notch1-Fc, Notch2-Fc, or Notch3-Fc by ELISA. MHN1-519, MHN2-25, and MHN3-21 reacted with CHO(r) cells (23) expressing human Notch1, Notch2, and Notch3, respectively, as demonstrated by flow cytometry (supplemental Fig. S4*A*[10]). MHN1-519 and MHN3h21 blocked Notch1-Fc and Notch3-Fc binding to CHO(r) cells expressing human Delta4, respectively, but MHN2-25 did not block Notch2-Fc binding (supplemental Fig. S4*B*).

[10] The online version of this article contains supplemental material.

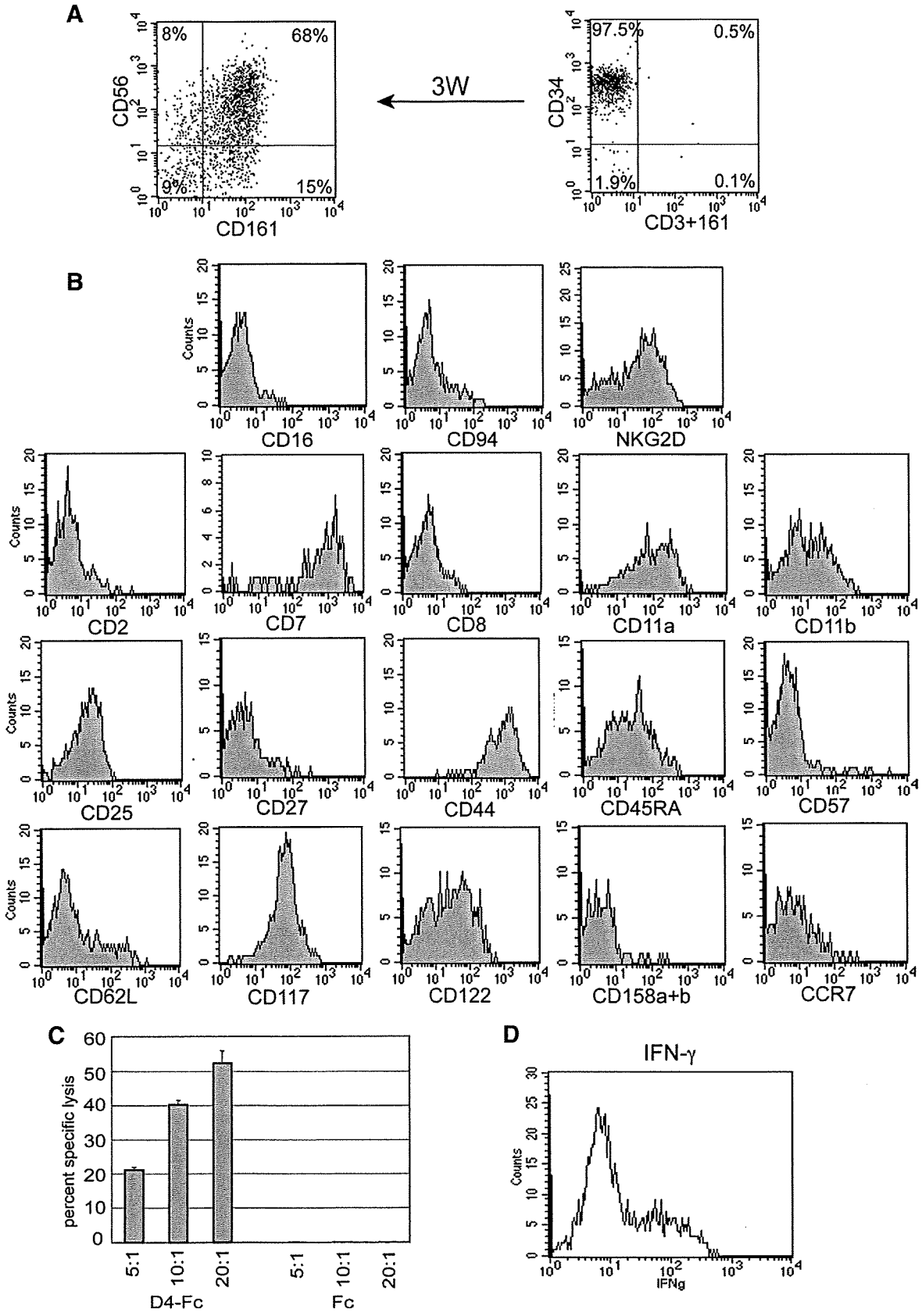

FIGURE 1. Phenotypic and functional analysis of cells derived from $CD34^+$ cells on Delta4-Fc-coated plates. *A*, Representative dot plot illustrating CD161 vs CD56 expression in the cells generated on Delta4-Fc-coated plates from $CD34^+$ CB cells after culture for 3 wk, and dot plot illustrating CD161/CD3 vs CD34 of the sorted CB population before culture. *B*, Various phenotypic analyses of the 3-wk cultured cells that were gated on $CD161^+$ events. Results are representative of at least four experiments. *C*, The 2.5-wk cultured cells were cytotoxic against K562 target cells at the indicated E:T ratios. The ratio of $CD161^+$ cells cultured on Delta4-Fc-coated plates and those Fc-coated plates in this experiment was 40 and 0%, respectively. Results are representative of four experiments. *D*, IFN-γ production by the 3-wk culture cells, as analyzed by intracellular expression. The histogram plots were gated on $CD56^+$events. Results are representative of five experiments.

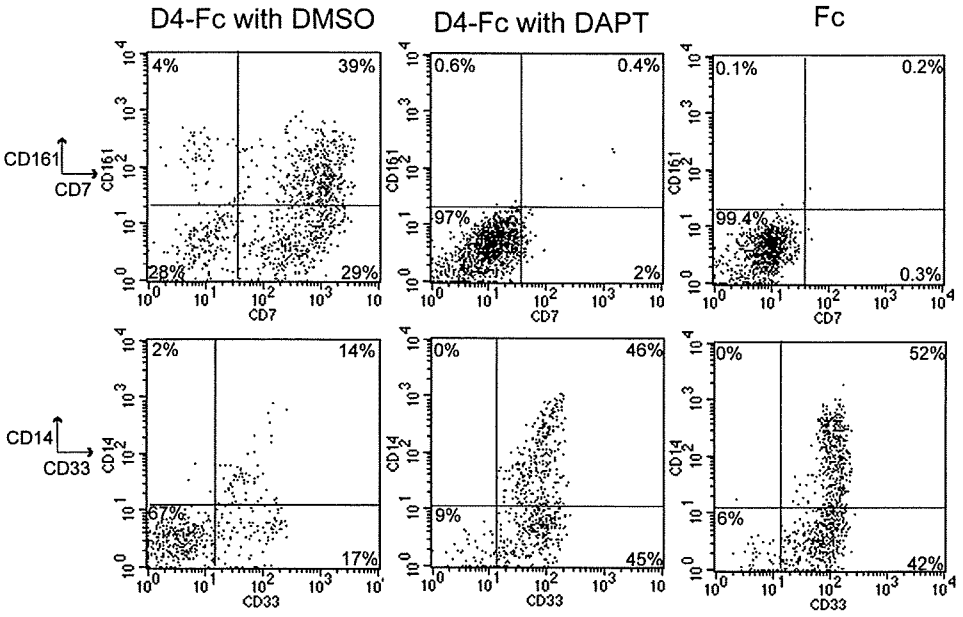

FIGURE 2. Phenotypic analysis of cells cultured in the presence of γ-secretase inhibitors. Representative dot plots of CB CD34$^+$ cells that were cultured for 2.5 wk on Delta4-Fc-coated plates with DMSO (the solvent for the γ-secretase inhibitors: D4-Fc with DMSO), Delta4-Fc-coated plates with DAPT (D4-Fc with DAPT), and Fc-coated plates (Fc). Results are representative of three experiments.

Results

Human CB CD34$^+$ and CD133$^+$ cells gave rise to functional NK cells by Notch signaling in a stroma-free culture without exogenous IL-15

CD34$^+$ or CD133$^+$ cells were cultured on Delta4-Fc-coated plates. The cells became almost immunophenotypically homogeneous after culture for ~3 wk (Fig. 1*A*). The proliferation efficiency depended on CB batches; fold increases in the cell number after the 3-wk culture were 10.3 ± 7.74-fold (n = 11). These cells expressed CD56 and CD161, but did not express surface CD3 or TCRα/β (data not shown). CD56/CD161 double-positive cells also expressed NKG2D and CD117, but were essentially negative for CD16 and killer Ig-like receptors (CD158a and CD158b). The cells had cytotoxic activity against K562 (Fig. 1*C*) and Jurkat cells (see Fig. 5*Bii*), and secreted IFN-γ (Fig. 1*D*). These results indicate that the culture products meet the general criteria for functional NK cells. The products generated from CB CD34$^+$ and CD133$^+$ had the same characteristics (data not shown).

Virtually no NK cells developed in culture on control Fc-coated plates; the vast majority of the cells were CD33$^+$ myeloid cells, a significant part of which expressed CD14 (Fig. 2). The absolute cell numbers with control Fc are ~5-fold higher than that with Delta4-Fc, and the fold increases in the cell number after the 3-wk culture were 45.7 ± 31.6-fold (n = 11). To confirm that the NK cell differentiation was Notch dependent, we added a γ-secretase inhibitor, DAPT, which strongly inhibits ligand-dependent Notch activation (24, 25). The cells cultured on Delta4-Fc-coated plates in the presence of DAPT had the same immunophenotype as those cultured on the control Fc-coated plates and did not give rise to NK cells (Fig. 2), indicating that the observed NK cell development was Notch activation dependent. The number of cells generated increased to the level of that in the control Fc protein-coated plates (data not shown).

We cultured CD34$^+$ cells and CD133$^+$ cells purified from G-CSF-mobilized peripheral blood cells. Both cell types gave rise to CD56$^+$CD161$^+$ NK cells that were similar to those derived from CB CD34$^+$ or CD133$^+$ cells. The amount of time required for mobilized peripheral blood CD34$^+$ or CD133$^+$ cells (~5 wk) to develop to a major population of CD56$^+$CD161$^+$ NK cells was greater than that required for CB CD34$^+$ or CD133$^+$ cells (supplemental Figs. S1*A* and S2 and Fig. 3), although the time courses varied to some degree from batch to batch (supplemental Fig. S2 and data not shown).

We next examined the effects of other soluble Notch ligands, human Delta1-Fc and Jagged1-Fc, on NK cell development from CB CD34$^+$ cells. Delta1-Fc had an effect similar to that of Delta4-Fc, although with lower efficiency (supplemental Fig. S1*B*), and Jagged1-Fc showed no potential to induce NK cell development (data not shown). Therefore, we used Delta4-Fc as the soluble Notch ligand and CB CD34$^+$ cells as the starting material for the remaining experiments.

IL-15 is dispensable for in vitro NK cell development from CB CD34$^+$ cells in the presence of Delta4 stimulation, whereas Notch stimulation appears to be essential for physiologic NK cell development

When IL-15 was added to the culture medium on control Fc-coated plates, CD56$^+$CD161$^+$ NK cells emerged (Fig. 3 and supplemental Fig.S2, Fc plus IL-15; cf with Fig. 3 and supplemental Fig.S2, Fc); this effect was blocked by anti-IL-15- neutralizing Ab (Fig. 3 and supplemental Fig.S2, Fc plus IL-15 plus anti-IL-15). IL-15 does not affect the absolute cell number; fold increases in the cell number after the 3-wk culture were 46.8 ± 36.3-fold, 43.1 ± 35.7-fold, and 48.4 ± 9.48-fold with IL-15 (n = 7), without IL-15 (n = 7), and with IL-15 and anti-IL-15 (n = 3) in the control Fc-coated plate condition. The rate of NK cell development by IL-15 stimulation, however, was much slower than that by Delta4-Fc stimulation. In the absence of Notch stimulation, but with IL-15, the percentage of total NK-lineage cells represented by positive CD161 was only 2.6 ± 2.9%, 6.3 ± 4.6%, and 9.0 ± 4.5% at 2, 3, and 4 wk, respectively (Fig. 3 and supplemental Fig.S2, Fc plus IL-15); whereas in Delta4-Fc with IL-15 (Fig. 3 and supplemental Fig.S2, D4-Fc plus IL-15) or without IL-15 (Fig. 3 and supplemental Fig.S2, D4-Fc), the percentage of total NK-lineage cells was 56 ± 17%, 77 ± 11%, and 81 ± 5.8% (with IL-15) or 52 ± 18%, 74 ±

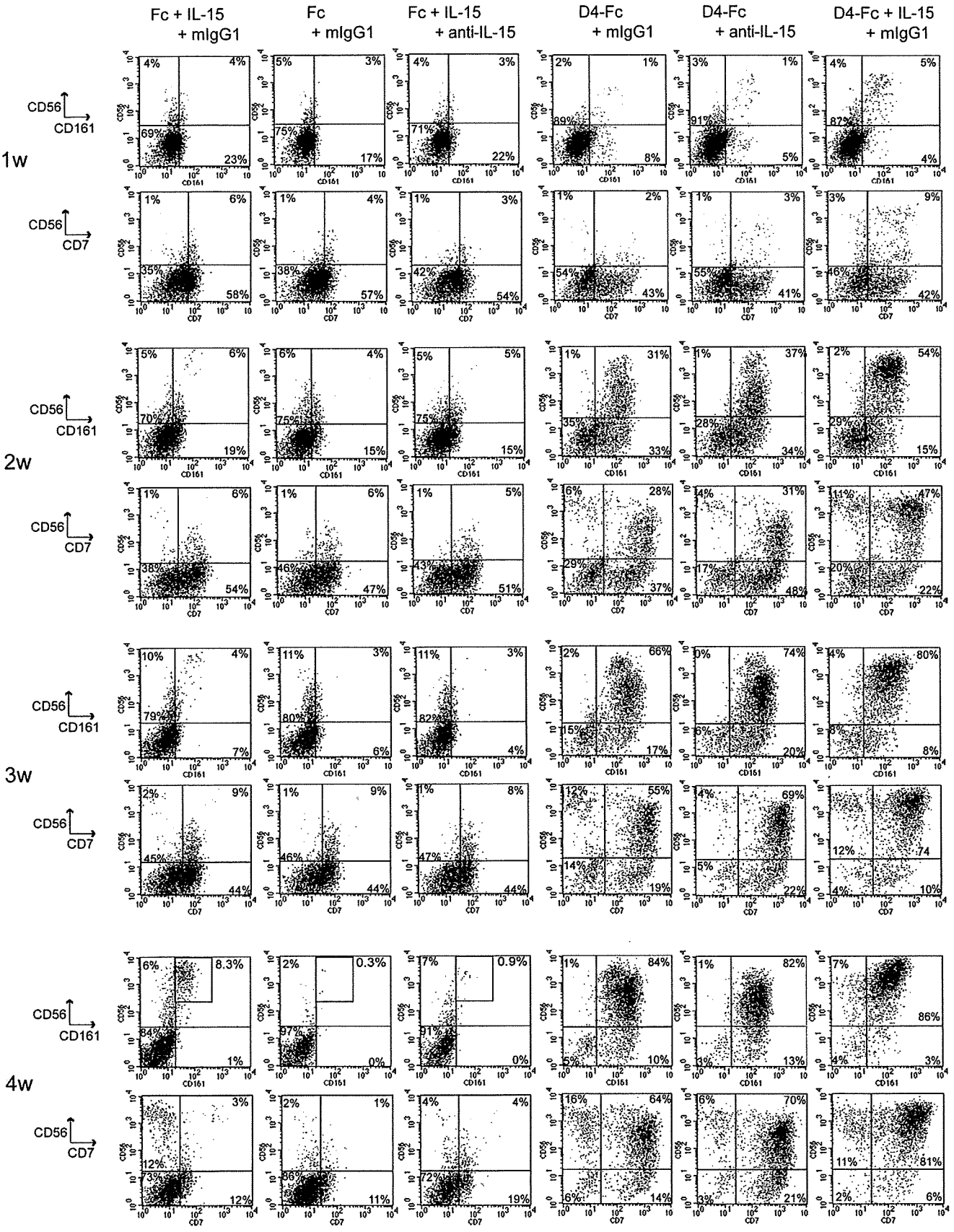

FIGURE 3. Phenotypic analysis during culture under several culture conditions. Representative dot plots illustrating CD161 vs CD56 and CD7 vs CD56 of cells that were cultured from CB CD34$^+$ cells for the indicated number of weeks on Fc-coated plates with IL-15 and mouse (m) IgG1-containing medium (Fc + IL-15 + mIgG1), Fc-coated plates with mouse IgG1-containing medium (Fc + mIgG1), Fc-coated plates with anti-IL-15 Ab-containing medium (Fc + anti-IL-15), Delta4-Fc-coated plates with mouse IgG1-containing medium (D4-Fc + mIgG1), Delta4-Fc-coated plates with anti-IL-15 Ab-containing medium (D4-Fc + anti-IL-15), and Delta4-Fc-coated plates with IL-15 and mouse IgG1-containing medium (D4-Fc + IL-15 + mIgG1). Results are representative of at least three experiments. The means and SD of each CD161 vs CD56 quadrant in replicate experiments are shown in supplemental Fig. S2.

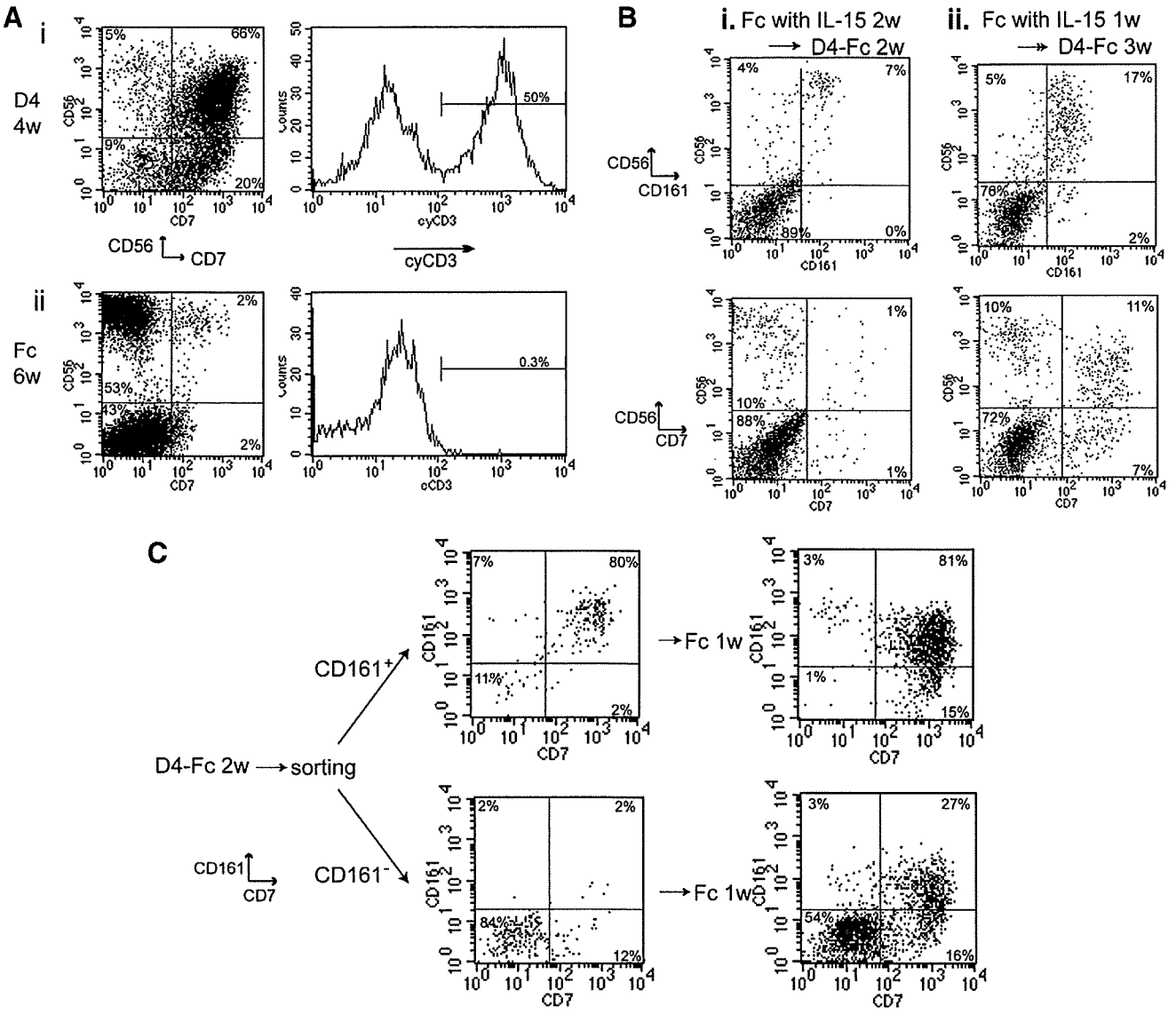

FIGURE 4. Phenotypic analysis of cells after various culture conditions. *A*, Representative dot plots illustrating CD7 vs CD56 cells that were cultured from CB CD34$^+$ cells for 4 wk on Delta4-Fc-coated plates (D4, *Ai*) and for 6 wk on Fc-coated plates in the presence of IL-15 (Fc, *Aii*). Histogram plots illustrating cyCD3 of the same cells that were gated on CD56$^+$ events. Results are representative of six and five experiments, respectively. *B*, Representative dot plots of cells that were cultured from CB CD34$^+$ cells for 2 or 1 wk on Fc-coated plates with IL-15-containing medium and were then transferred to Delta4-Fc-coated plates and cultured for 2 or 3 wk, respectively, with IL-15-free medium (*Bi* and *Bii*). Results are representative of three experiments. *C*, Representative dot plots illustrating CD7 vs CD161 expression in the cells that were sorted into CD161$^+$ or CD161$^-$ after 2-wk culture from CB CD34$^+$ cells on Delta4-Fc-coated plates, and dot plots of cells that were cultured another week on Fc-coated plates with IL-15-free medium. Results are representative of three experiments.

11%, and 88 ± 6.7% (without IL-15) at 2, 3, and 4 wk, respectively. (supplemental Fig. S2*Bi*) The differences were statistically significant between the D4-Fc group and the Fc group ($p < 0.001$). The adjusted absolute numbers of NK-lineage cells cultured on Delta4-Fc tended to be greater than those cultured on Fc with IL-15, although the differences were not always statistically significant (supplemental Fig. S3*C*). CD56$^+$ CD161$^+$ NK cells eventually comprised a major population after 6 wk of culture with IL-15 but without Notch stimulation (Fig. 4*Ai*). No CD56$^+$CD7$^+$ (Fig. 3, Fc plus IL-15) or CD56$^+$ cyCD3$^+$ (Fig. 4*Aii*) cells were detected during culture with IL-15 but without Delta4-Fc, whereas Delta4-Fc stimulation induced the generation of CD7$^+$cyCD3$^+$ cells, which could represent naturally arising T/NK cell progenitors (26, 27), at the early phase of the culture. Although CD7^{low} cells appeared in culture with IL-15 alone, they might represent monocytes, because a substantial amount of CD14$^+$ cells emerged regardless of the presence of IL-15 when Delta4-Fc was absent and peripheral blood monocytes express CD7 at low levels.

Delta4-Fc stimulation without IL-15 efficiently induced NK cell development (Figs. 1 and 3 and supplemental Fig. S2, D4-Fc). Most of the cells became CD7^{high} in the first 2 wk. A few CD161$^+$ cells were detected at the first week, the number of which increased at the next week. Only a part of the CD161$^+$ cells was positive for CD56 during the early phase of the culture, but at the later time points, most CD161$^+$ cells were CD56$^+$. This observation may indicate that CD161$^+$CD56$^-$ cells emerge at first and they gradually become CD161$^+$ CD56$^+$, although there is another interpretation such as simultaneous generation of double-positive and CD161 single-positive cells, expansion of double-positive cells, and apoptotic disappearance of the single-positive cells. Given the previous

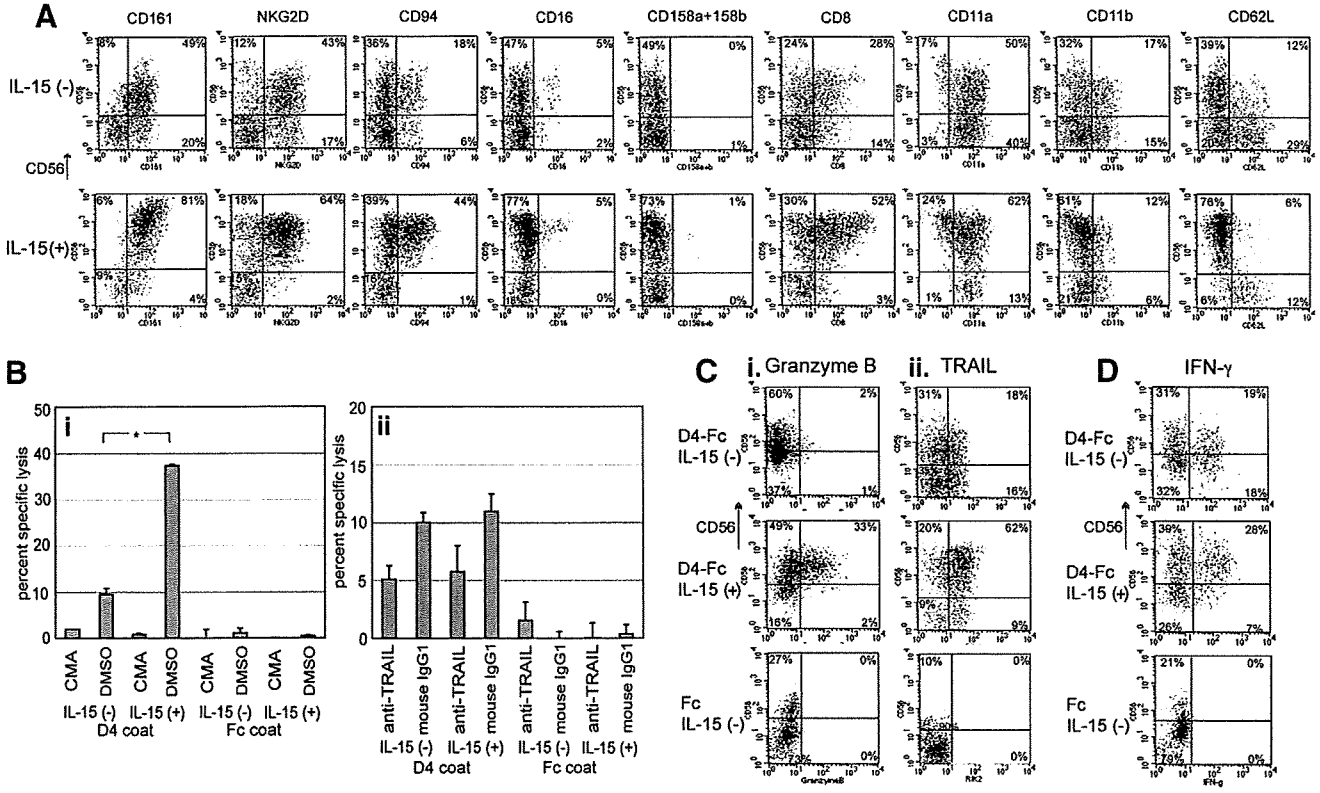

FIGURE 5. Phenotypic and functional differences between cells cultured in IL-15-containing and IL-15-free medium on Delta4-Fc-coated plates. *A*, Representative dot plots illustrating CD56 vs indicated Ags of cells cultured for 3 wk from CB CD34$^+$ cells in IL-15-containing or IL-15-free medium on Delta4-Fc-coated plates. Results are representative of six experiments. *B*, Cytotoxicity against K562 (*Bi*) or Jurkat (*Bii*) target cells at an E:T ratio of 5:1. Effectors were developed in the indicated conditions for 2.5 wk. In this experiment, the ratio of CD161$^+$ cells cultured on Delta4-Fc-coated plates with or without IL-15 condition and those cultured on Fc-coated plates with or without IL-15 condition were 53, 46, 0.6, and 0%, respectively. Effectors were pretreated with CMA or DMSO (the solvent for CMA) (*Bi*). Anti-TRAIL RIK-2 or its isotype control mouse IgG1 was added at the start of the cytotoxicity assay (*Bii*). Results are representative of three (*Bi*) and six (*Bii*) experiments. Batch to batch variation can be seen by comparing this figure with Fig. 1. *C*, Representative dot plots illustrating intracellular granzyme B (*Ci*) or TRAIL (*Cii*) vs CD56 of the cells cultured for 3 wk in medium with or without IL-15 on Delta4-Fc-coated plates and without IL-15 on Fc-coated plates. Results are representative of four experiments. *D*, Representative dot plots illustrating intracellular IFN-γ vs CD56 of cells cultured for 3 wk in medium with or without IL-15 on Delta4-Fc-coated plates and without IL-15 on Fc-coated plates. Results are representative of four experiments.

demonstration that CD161 is expressed on the cell surface earlier than CD56 (28), the former possibility appears more likely. To explore the possibility that IL-15 is secreted by a certain population of cells during culture and contributes to NK cell development, we added anti-IL-15-neutralizing Ab to the culture. The addition of anti-IL-15-neutralizing Ab to the culture medium blocked NK cell development in the presence of IL-15 (Fig. 3, IL-15 plus anti-IL-15), but did not affect either the rate or efficiency of Delta4-Fc-dependent NK cell emergence (Fig. 3, D4-Fc plus anti-IL-15, fold increase in the cell number after 3-wk culture on Delta4-coated plate with anti-IL-15 was 8.75 ± 4.18-fold (n = 5), which was not statistically different from those cultured on Delta4-coated plates without anti-IL-15 or with IL-15), further supporting the possibility that IL-15 is dispensable for NK cell development from human CB CD34$^+$ cells.

IL-2 is also suggested to be involved in the NK cell development. To examine whether IL-2, which might be secreted by a certain population of the cells, was present in the culture, the IL-2 concentration in the supernatant was measured by ELISA. No IL-2 was detected (cutoff level, 7 pg/ml; data not shown), indicating that IL-2 was not involved in the NK cell development induced by Delta4-Fc.

To examine the NK cell developmental stages that are critically dependent on Notch signaling, we cultured CB CD34$^+$ cells on control Fc-coated plates with IL-15 for 1 or 2 wk and then transferred them onto Delta4-Fc-coated plates and cultured them further for 3 or 2 wk without IL-15, respectively (culturing for a total of 4 wk). Approximately 50% of the CD56$^+$ CD161$^+$ population expressed CD7$^+$ at 4 wk in the 1-wk IL-15 condition (Fig. 4*Bii*). In contrast, very few CD56$^+$ cells that emerged in the 2-wk IL-15 condition expressed CD7 (Fig. 4*Bi*). These observations indicated that CB CD34$^+$ cells cultured with IL-15, but without Notch stimulation, for 1 wk retained the capacity to generate CD56$^+$CD7$^+$ cells, but that they lost this capacity when cultured without Notch stimulation for 2 wk. We also examined whether the Notch stimulation at early phases of the culture irreversibly determines NK cell developmental fate. To examine the early phase of NK cell development, we cultured CB CD34$^+$ cells for 2 wk on Delta4-Fc-coated plates and sorted the product into CD161$^+$ and CD161$^-$ cells, because CD161 is known to be expressed earlier than CD56 on the cell surface (28). We then transferred each population onto control Fc-coated plates and cultured them for another week without IL-15. More than 80% of the population derived from the CD161$^+$ cells expressed CD7$^+$. Interestingly, the CD161$^-$ cells also gave rise to CD161$^+$CD7$^+$ cells among one of the two major populations (Fig. 4*C*). These observations indicate that Notch activation irreversibly drives a subset of CD34$^+$ cell

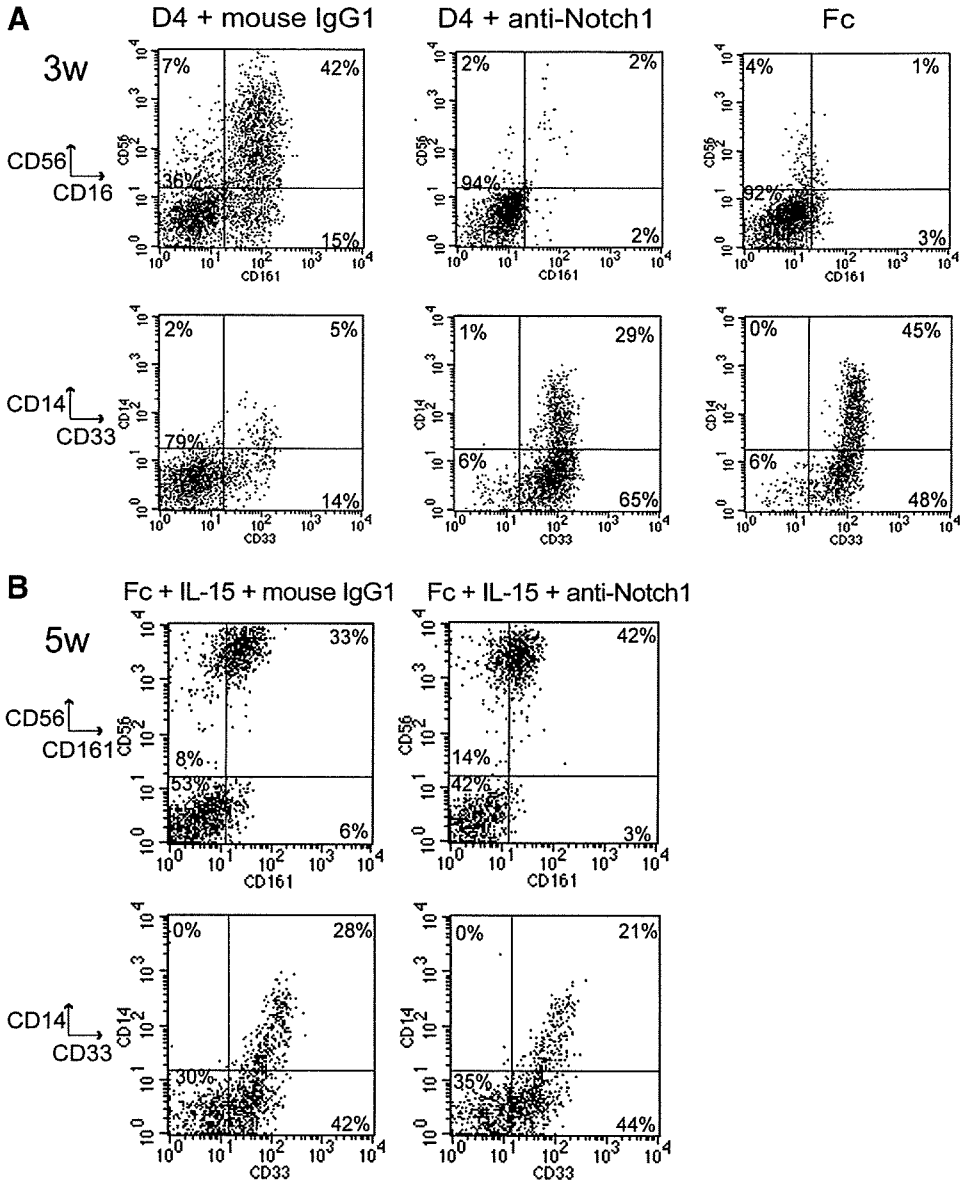

FIGURE 6. Phenotypic analysis of cells cultured in the presence of human Notch1-blocking Ab. *A*, Representative dot plots of cells that were cultured for 3 wk from CB $CD34^+$ cells on Delta4-Fc-coated plates with mouse IgG1-containing medium, Delta4-Fc-coated plates with anti-human Notch1-containing medium, and Fc-coated plates. Results are representative of six experiments. *B*, Representative dot plots of cells that were cultured for 5 wk from CB $CD34^+$ cells on Fc-coated plates with IL-15 and mouse IgG1-containing medium and Fc-coated plates with IL-15 and anti-human Notch1-containing medium. Results are representative of three experiments.

progenies to the $CD161^+CD7^+$ NK cell fate within 2 wk, presumably before $CD161^+$ is expressed.

IL-15, along with Delta4 stimulation, induces phenotypic maturation and functional augmentation of CB $CD34^+$ cell-derived NK cells

We compared the immunophenotype of the CB $CD34^+$ cell-derived NK cells generated in the culture with Delta4-Fc but lacking IL-15 (D4-Fc) and in culture with Delta4-Fc and IL-15 (D4-Fc plus IL-15). IL-15 does not affect the absolute cell number; fold increases in the cell number after the 3-wk culture were 10.6 ± 6.16-fold and 10.2 ± 6.71-fold with and without IL-15 in the D4-Fc-coated plate condition (n = 8). The cells grew slightly faster with D4-Fc plus IL-15 than with D4-Fc alone, but there were no significant differences in the frequency of $CD56^+CD161^+$ population in both conditions after 3 wk (cf Fig. 3 and supplemental Fig. S2*A*, D4-Fc and D4-Fc plus IL-15; supplemental Fig. S2*Bii*; and Fig. 5). The expression levels of CD7 and NKG2D were similar. CD94 was expressed at a higher level in the D4-Fc plus IL-15 condition. CD16 and CD158 were not expressed in the D4-Fc condition, but were expressed at low levels in the D4-Fc plus IL-15 condition. The expression levels of adhesion molecules, i.e., CD11a, CD11b, and CD62L, were higher in the D4-Fc condition (Fig. 5*A*). The other markers shown in Fig. 1 (CD2, CD7, CD25, CD27, CD44, CD45RA, CD57, CD117, CD122, and CCR7; data not shown), as well as IFN-γ (Fig. 5*D*), were expressed at similar levels under both conditions. There was a remarkable difference in the expression level of CD56, which was markedly higher in the D4-Fc plus IL-15 condition.

Cytotoxic activity against K562 cells was significantly higher in NK cells generated in the D4-Fc plus IL-15 condition than that in the D4-Fc condition. CMA, an inhibitor of perforin-mediated cytotoxicity, had a stronger suppressive effect on the cytotoxic activities of NK cells generated in the D4-Fc plus IL-15 condition (Fig. 5*Bi*). Interestingly, granzyme B, which enhances the perforin-mediated cytotoxicity and whose expression was not detected in the D4-Fc condition, was up-regulated in the D4-Fc plus IL-15 condition (Fig. 5*Ci*). This might explain the stronger suppression of NK cell cytotoxic activity by CMA when generated in the D4-Fc plus IL-15 condition compared with the D4-Fc condition. In contrast, there was no significant difference in the killing activities against Jurkat cells of the NK cells generated under