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Ⅲ　分担研究報告

厚生労働省科学研究補助金（難治性疾患克服研究事業）
分担研究報告書

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研究要旨

RCAN1 は calcineurin の阻害分子として知られているが、白血病細胞を始めとする造血腫瘍細胞で高発現していることが見いだされた。RCAN1 は癌抑制蛋白と結合し、血液細胞の生存・増殖に関与していると思われる。慢性好中球減少症における RCAN1 の発現および機能について検討を進めている。

A．研究目的

慢性好中球減少症における好中球減少の分子メカニズムを、RCAN1 分子に着目して明らかにする。

B．研究方法

1. ヒト白血病細胞株、ヒト白血病患者骨髄細胞および正常骨髄細胞における RCAN1 の発現量を比較検討する。
2. ヒト RCAN1 遺伝子プロモーター活性の調節機序を、白血病細胞株を用いたルシフェラーゼアッセイ法により解析する。
3. RCAN1 結合因子を yeast two hybrid 法を用いて同定し。RCAN1 下流のシグナルを明らかにする。
4. 慢性好中球減少症患者の骨髄細胞における RCAN1 の発現量を検討する。
5. 慢性好中球減少症患者における、RCAN1 下流シグナルの活性について検討する。

C．研究結果

1. 16 例中 15 例の急性骨髄性白血病(AML)患者の骨髄単核細胞で、RCAN1 の高いレベルの発現を認めた。また、検討した全てのヒト骨髄性白血病細胞株（KCL22, K562, KU812, THP-1, U937, HL60, KY821)においても、高い RCAN1 の発現を認めた。

2. ヒト正常骨髄単核細胞（米国 Lonza Walkersville 社より購入）では、CD34(+)CD38(-)（造血幹細胞), CD34(+)CD38(+)（造血前駆細胞), CD33(+)CD14(-)CD16(-)（骨髄系前駆細胞), CD33(+)CD14(+)（単球/マクロファージ系前駆細胞)および CD33(+)CD16(+)（顆粒球系前駆細胞)の全ての細胞分画において、白血病細胞と比較して RCAN1 の発現量は低下していた。

3. RCAN1 shRNA を AML 細胞株 HL60 に導入して RCAN1 発現を抑制すると、メ

チルセルロース培地上でのコロニー形成能が著明に低下した。同様に RCAN1 の発現を抑制した HL60 細胞は、低血清培地において cleaved caspase 3、cleaved caspase 7、cleaved caspase 9、cleaved PARP 等の増加を認めた。

4. ヒト RCAN1 遺伝子プロモーターの機能解析の結果、RCAN1 の発現に重要なプロモーター領域が、—459 ～ —249 bp に存在するが明らかとなった。

5. K562 細胞遺伝子ライブラリーを用いた two-hybrid assay 法により、RCAN1 は癌抑制蛋白として知られる HINT1, HINT2 および LINT1 と結合することを見出した。

D．考察

RCAN1 発現量が、正常骨髄細胞では低い一方で、ほとんどの骨髄性白血病細胞では高かったことから、AML など増殖の亢進している血液細胞で高い可能性がある。この結果を支持する結果として、急性リンパ性白血病細胞株や多発性骨髄腫など他の造血器腫瘍細胞においても RCAN1 の高発現が認められている。さらに、shRNA による RCAN1 の発現抑制の結果および RCAN1 結合蛋白の同定により、RCAN1 がいくつかの癌抑制蛋白と結合することで、最終的に血液細胞の生存・増殖に関与していることが示唆された。このことから、慢性好中球減少症においては造血幹細胞～顆粒球系前駆細胞における RCAN1 の発現がさらに低下しており、それが好中球減少の一因になっている可能性が考えられる。現在、この仮説を検証するため、慢性好中球減少症患者の骨髄細胞における RCAN1、HINT1、HINT2、LINT1 の発現について検討する予定となっている。

E．結論

1. RCAN1 は、正常造血細胞では発現量が低く造血腫瘍細胞で高発現している。
2. RCAN1 は、癌抑制蛋白への干渉を介して血液細胞の生存・増殖に関与している可能性がある。
3. 慢性好中球減少症の分子機序を理解するため、 RCAN1 の発現およびその役割について検討する予定である。

F．研究危険情報

なし

G．知的財産権の出願・登録状況

1．特許取得

なし

2．実用新案登録

なし

3．その他

なし

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分担研究報告書

「多施設参加型研究における円滑な倫理審査をサポートするためのシステム作成」の研究

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研究要旨

多施設共同研究では研究開始にあたり、個々の参加施設で倫理審査・承認が必要とされることが多い。この手続きにより、施設によっては研究承認が遅れ、研究遅延の一因になっていると考えられる。本研究では、多施設共同研究をスムースに進展させるための蓄積を目的として、これまでの国内の研究倫理審査の状況を把握し、問題点を抽出整理し、新たな調査計画を作成した

A．研究目的

多施設共同研究では研究開始にあたり、多くの場合個々の研究参加施設で倫理審査・承認が必要とされることが多い。この手続きは労力・時間を要するものであり、施設によっては研究承認が遅れ、研究遅延の一因になっていると考えられる。

本研究は、施設毎の倫理審査、特に研究倫理審査の状況を把握し、問題点を抽出し、倫理委員会書類作成のサポートシステムを構築し、多施設共同研究をスムースに進展させるための蓄積を試みることを目的とした。

B．研究方法

倫理審査についての先行研究を、国内のデータベースを元にレビューした。データベースとしては、医中誌 WEB (http://www.jamas.or.jp/index.html)、および JDreamII (http://pr.jst.go.jp/jdream2/) を用いた。またレビューした結果から、現状の問題点を更に抽出することを目的とした調査計画を作成した。

C．研究結果

医中誌および JDreamII のデータベースを用い、「倫理審査」をキーワードに文献検索を行った。その結果 49 件の原著論文、総説を入手しレビューした。その中で研究倫理審査の実態調査は 1990 年以後 9 報であった。この内、平成 15 年の厚生労働省「臨床研究に関する倫理指針」制定後（平成 16 年度より施行）の実態調査は 5 件で、平成 20 年の全部改正後（平成 21 年度より施行）の実態調査は、検索されなかった。

D．考察および調査計画

レビュー結果から、国内の研究倫理審査の主な問題点として以下の 6 点があげられる。

1．国内の倫理審査委員会の数が無秩序に

増加し、倫理審査委員会の構成、審査方法、審査基準に施設間でばらつきがある。

2．倫理委員会自体の組織構成、審査方法において、相当数の委員会が倫理指針に抵触している。

3．審査に際して、審査基準が整備されていない。

4．承認後のフォローが十分になされていない。

5．審査委員の教育の機会がない。

6．各施設の倫理審査委員会において事務局体制など人的サポートが十分でない。

また既報から、上記の問題点解決のための提言として以下が要約される。

3．日本における倫理原則の確立とともに、包括的、系統的な統一倫理指針の作成

4．中央審査機関の設置、あるいは倫理審査委員会の登録、認定制などの創設により審査の質の標準化を行う。

実際、新たな試みとして、倫理審査委員会の登録や、e-learning の導入による倫理審査委員会の質向上の取り組み（福岡臨床研究倫理審査委員会ネットワーク）も始まっている。このようなネットワークが発達することにより、今後統一倫理指針の作成や、中央審査制度の創設への期待も持てる。また国際的にみても、韓国では共同臨床試験審査委員会が導入され、IRB の相互承認などもなされている。

一方、先行研究により倫理審査委員会が抱える課題はある程度抽出されているが、倫理審査を申請する研究者を対象とした実態調査はなされていない。また、平成 21 年度より施行された臨床研究倫理指針では、被験者に対する補償など新たな改正点があるが、研究者側がこれらの変更点について理解し対応しているかについても疑問である。これらの点を踏まえ、倫理審査申請者を対象とした実態調査を行うことは意義ある事と考え、新たな調査計画を作成した。

調査計画

1）調査対象、調査法

研究者を対象に倫理審査申請する場合の実態について調査する。また、施設種別、規模により審査内容が異なる事も予想されるため、病床数などのデータを収集するほか、施設毎の審査申請書のフォーマットを可能な場合提供してもらう。

3）　調査対象の倫理指針

倫理審査申請する研究の種類により、研究者の抱える問題点は異なると思われるが、今回は殆どの施設が関わっていると予想される、「臨床研究に関する倫理指針」、「疫学研究に関する倫理指針」に対応する研究申請に際しての実態調査とする。

3）質問表の骨子

（1）主として研究倫理審査の構造に関わるもの

○施設種、施設規模

○施設内倫理審査委員会設置の有無、組織構成

○倫理審査申請に関する相談窓口の有無

○倫理審査委員会の開催頻度

（2）主として研究倫理審査申請のプロセスに関わるもの

○研究倫理指針、その他必要な知識について、研究者への教育研修の有無

○審査申請文書を作成する時間の確保状況
○申請書書式
○申請文書作成の際の人的サポートの有無
○研究申請に適応する倫理審査指針の判断の適切性
○健康被害に対する補償制度の認知度
○軽微な審査、迅速審査制度の活用状況
○審査結果の公表状況
○利益相反についての審査状況
（3）研究倫理審査のアウトカムに関するもの
○　対象が実施している臨床研究の件数
○ 対象が行った倫理審査の申請件数
○ 倫理申請から審査結果を得るまでの期間
（5）　その他
（1）～（4）を骨子とした調査表を作成した。

E．結論

研究倫理審査のこれまでの問題点を整理し、研究申請者を対象とした調査計画を策定した。

F.　研究危険情報

なし

G. 知的財産権の出願・登録状況

1．特許取得

特になし

2. 実用新案登録

特になし

3. その他

特になし

H. 研究発表

1．論文発表　特になし

2．学会発表　特になし

厚生労働省科学研究補助金（難治性疾患克服研究事業）

成人における慢性好中球減少症(周期性好中球減少症、慢性本態性好中球減少症、自己免疫性好中球減少症など）に関する調査研究

分担研究報告書
小児期の好中球減少症

研究分担者　　福島　敬　（筑波大学小児科学講師）

研究要旨

好中球減少等の血球減少症は、３血球系統のうち単独にまたは複合的に生じ、同時発症であることも、時差発症であることもある。小児期には一過性のものが多いことは事実であるが、原因・自然歴は様々で、診断基準や病型分類、標準治療についても統一見解のないものが多い。日本小児血液学会では、小児血液難病の発生数を把握することを主な目的として疾患登録制度を整備した。従来から小児慢性特定疾患の事業による当該疾患に関連する医療費の全額支給をおもな目的として、血液難病患児の多くが、診断名や治療内容等の臨床情報を行政機関に提供してきたが、それとは別に、地方公共団体ごとに診断名に特定されない「マルフク」等の医療費全額支給制度が整備されるにつれ、小児慢性特定疾患の手続きを取らない患児の割合が増加傾向にあることも、学会による登録制度の必要度が増した背景である。インターネットでアクセス可能なサイトを作成し、個人情報を含まない範囲で新規症例ごとに入力・登録するものであるが、あくまでも主治医による自主登録であり、入力操作の支援体制がない段階であるため、必要最小限の項目のみに絞らざるを得ない。

疾患概念の整理と診断基準の整備・周知、登録率向上のための工夫等の課題が認識されているが、未来に遺せる学術的財産であることは明らかであり、更に集計によって得られる情報は、好中球減少を含めた血液難病に対する行政施策立案のためにも貢献できるものである。

A．研究目的

日本小児血液学会による疾患登録システムをユーザー（登録者）側から検証し、その有用性および課題を抽出する。

B．研究方法

以下のアドレスから、施設毎に発行されるパスワードを使用してログインし、対象疾患を新規診断症例毎に登録操作を行う。

http://www.jsph.info/osirase/JSPH-touroku.html

好中球減少症については以下の分類がなされている。本システムでは診断基準が提示されていないため、主治医毎に書籍を参考にしながら診断することになる。

現在の登録システムによる好中球減少症の分類は以下のとおりである。

（1）先天性

Kostmann 症候群

Shwachman 症候群

先天性その他

（2）後天性

①慢性良性好中球減少症

抗好中球抗体陽性（自己免疫性）

抗体不明

②周期性好中球減少症

③無顆粒球症

薬剤性

その他

原因不明

C．研究結果

われわれが「好中球減少症」と診断した小児例のうち、母親が有する自己抗体が経胎盤的に新生児に影響する血球減少、および母親が父親の抗原に感作されて産生された同種抗体が経胎盤的に移行して、父親と同じ抗原を有する新生児の血球に作用するもの（同種免疫性血球減少症）は、現在の分類では「③無顆粒球症、その他」に登録せざるを得ないが、病態を考慮すると、「①慢性良性好中球減少症、抗好中球抗体陽性」の中に、「自己免疫性」以外の分類（たとえば「移行抗体によるもの」など）を追加するのが望ましいと考えられた。

自己免疫性溶血性貧血や特発性血小板減少性紫斑病と前後して発症する自己免疫性好中球減少症、一過性の骨髄線維症を伴う自己免疫性汎血球減少症、自己免疫性血球減少が、自己免疫性造血前駆細胞減少症（＝特発性再生不良性貧血？）に進行した症例、後に全身性エリテマトーデスを発症した症例などの位置づけが不明確であり、登録者によって判断が異なる場合が多いのではないかと危惧された。

自己抗体検出の精度がラボラトリーによって異なることが、大きな問題である。たとえば抗好中球抗体は、A社による商業ベースの方法では、自験例では陽性の判定がなされたことがないが、H大学輸血部で研究として実施しているものでは、抗原の同定まで含めて高精度に結果報告を得ることができ、経時的に見ても臨床所見と整合性のとれた力価であった。更に、直接・間接クームス試験は陰性であっても、J大学で実施している高感度の手法をもって解析すると、PA－IgG と同じように定量値を持って高感度に検出可能であった。

D．考察

疾患登録制度発足後の数年間は、試験期間という役割もあると考えられるため、登録内容を検証したうえで必要な診断基準を整備する必要がある。更に入力作業の担当者に医師の資格は必須ではないと考えられるため、臨床試験における CRC またはメディカルクラーク等の養成と配置が期待される。

E．結論

日本小児血液学会による疾患登録制度が継続することによって、疾患発生状況の横断的・縦断的評価が可能になることが大きなメリットである。一方で、診断方法（手

技）の標準化、診断基準の整備と入力支援体制の構築とは、登録精度・登録率の向上のために必須である。

F．研究発表

1．論文発表

なし。

2．学会発表

なし。

H. 知的財産権の出願・登録状況

なし。

1．特許取得

なし。

2. 実用新案登録

なし。

3. その他

なし。

Ⅳ　研究成果刊行物・別刷り

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

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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.

www.jimmunol.org/cgi/doi/10.4049/jimmunol.0803036

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.

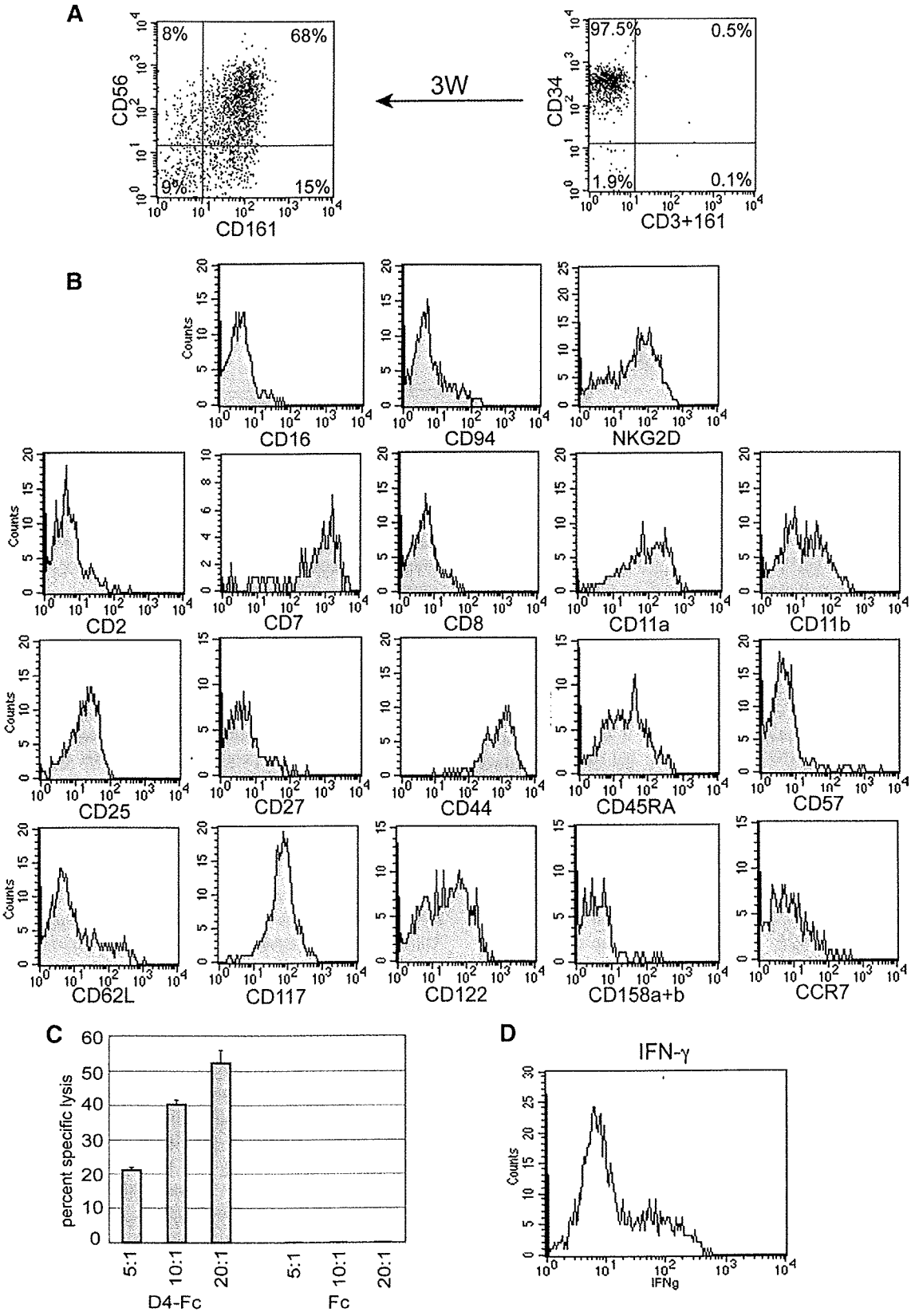

FIGURE 1. Phenotypic and functional analysis of cells derived from $CD34^+$ cells on Delta4-Fc-coated plates. *A*, Representative dot plot illustrating CD161 vs CD56 expression in the cells generated on Delta4-Fc-coated plates from $CD34^+$ CB cells after culture for 3 wk, and dot plot illustrating CD161/CD3 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.

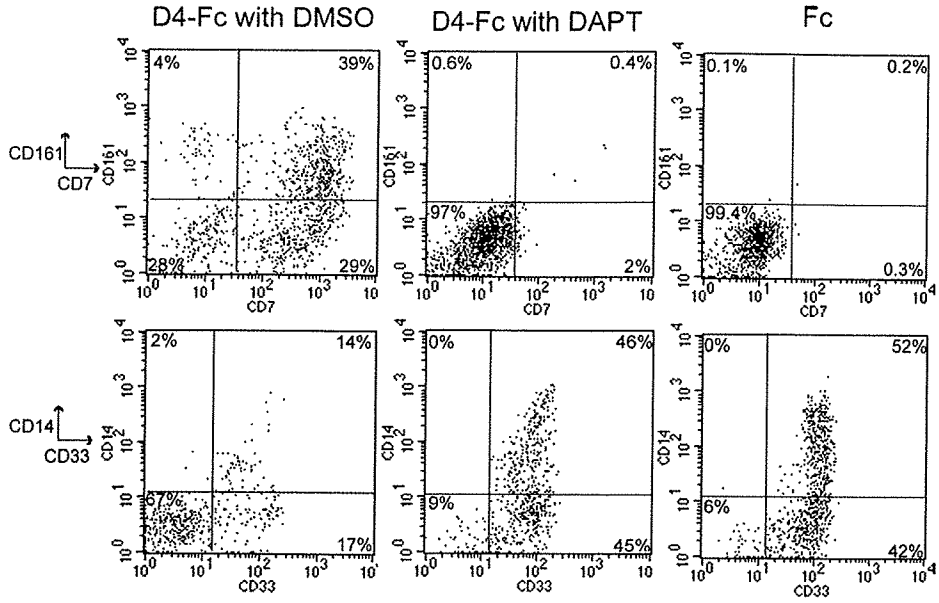

FIGURE 2. Phenotypic analysis of cells cultured in the presence of γ-secretase inhibitors. Representative dot plots of CB CD34$^+$ cells that were cultured for 2.5 wk on Delta4-Fc-coated plates with DMSO (the solvent for the γ-secretase inhibitors: D4-Fc with DMSO), Delta4-Fc-coated plates with DAPT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

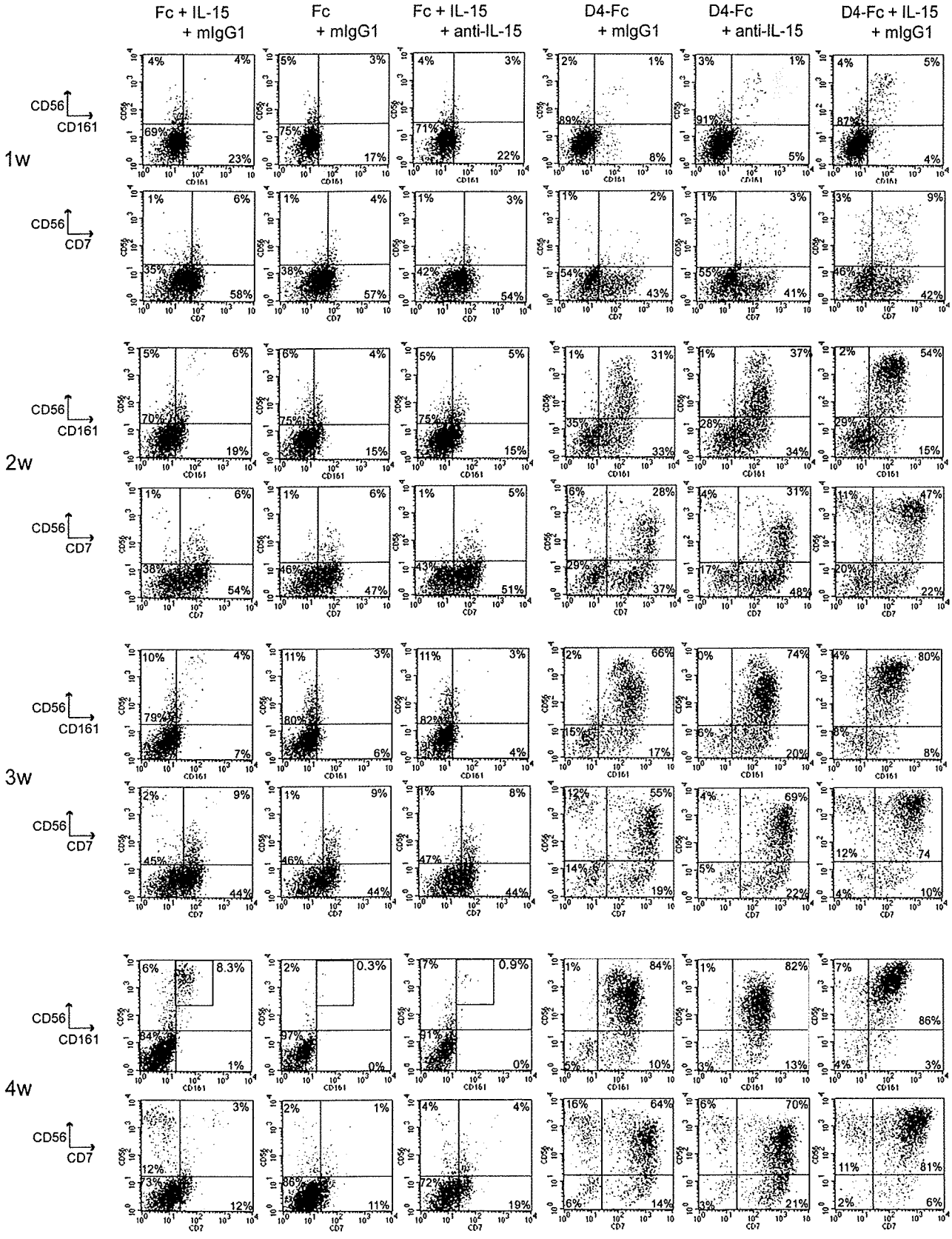

FIGURE 3. Phenotypic analysis during culture under several culture conditions. Representative dot plots illustrating CD161 vs CD56 and CD7 vs CD56 of cells that were cultured from CB $CD34^+$ cells for the indicated number of weeks on Fc-coated plates with IL-15 and mouse (m) IgG1-containing medium (Fc + 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.

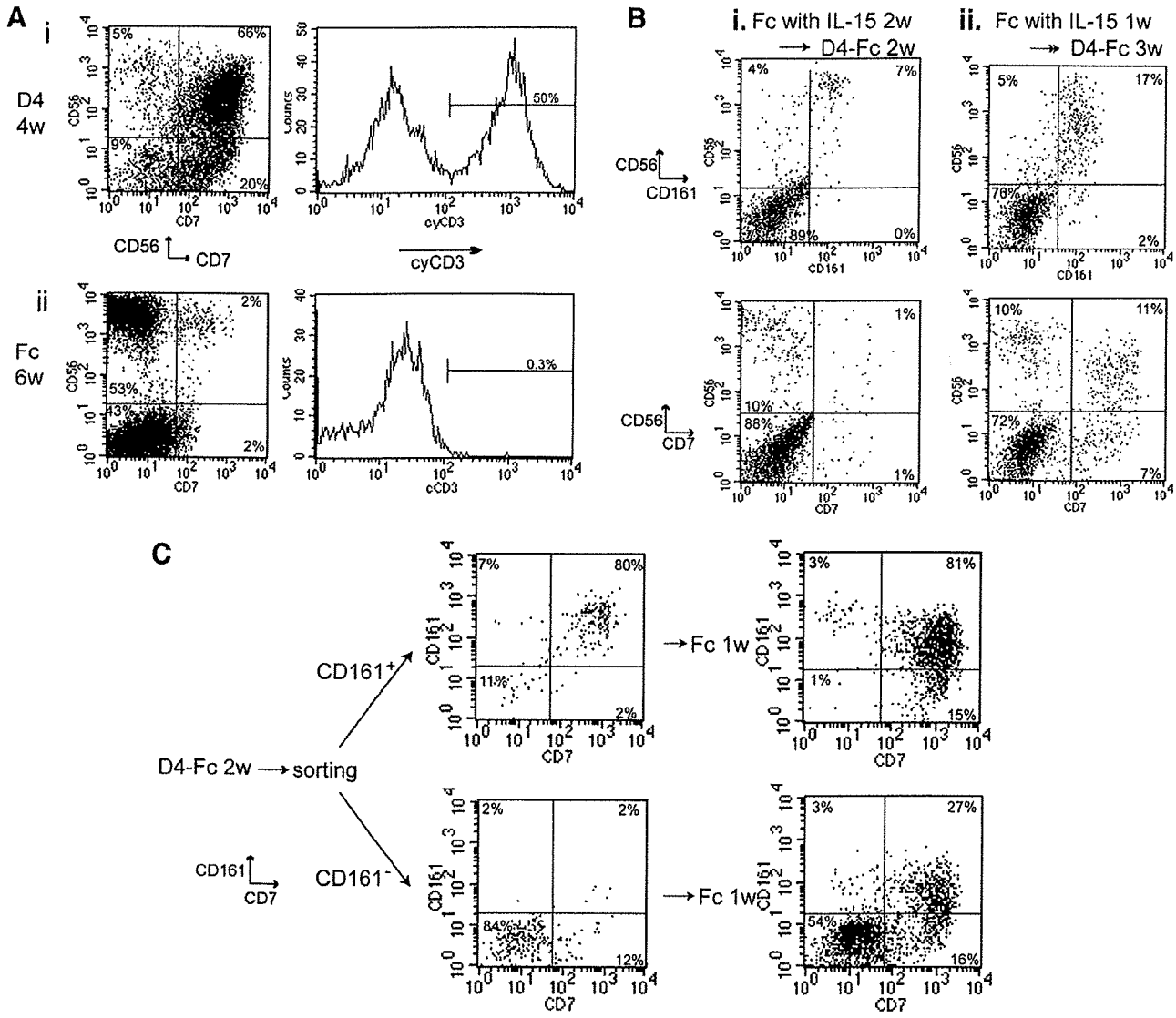

FIGURE 4. Phenotypic analysis of cells after various culture conditions. *A*, Representative dot plots illustrating CD7 vs CD56 cells that were cultured from CB $CD34^+$ cells for 4 wk on Delta4-Fc-coated plates (D4, *Ai*) and for 6 wk on Fc-coated plates in the presence of IL-15 (Fc, *Aii*). Histogram 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

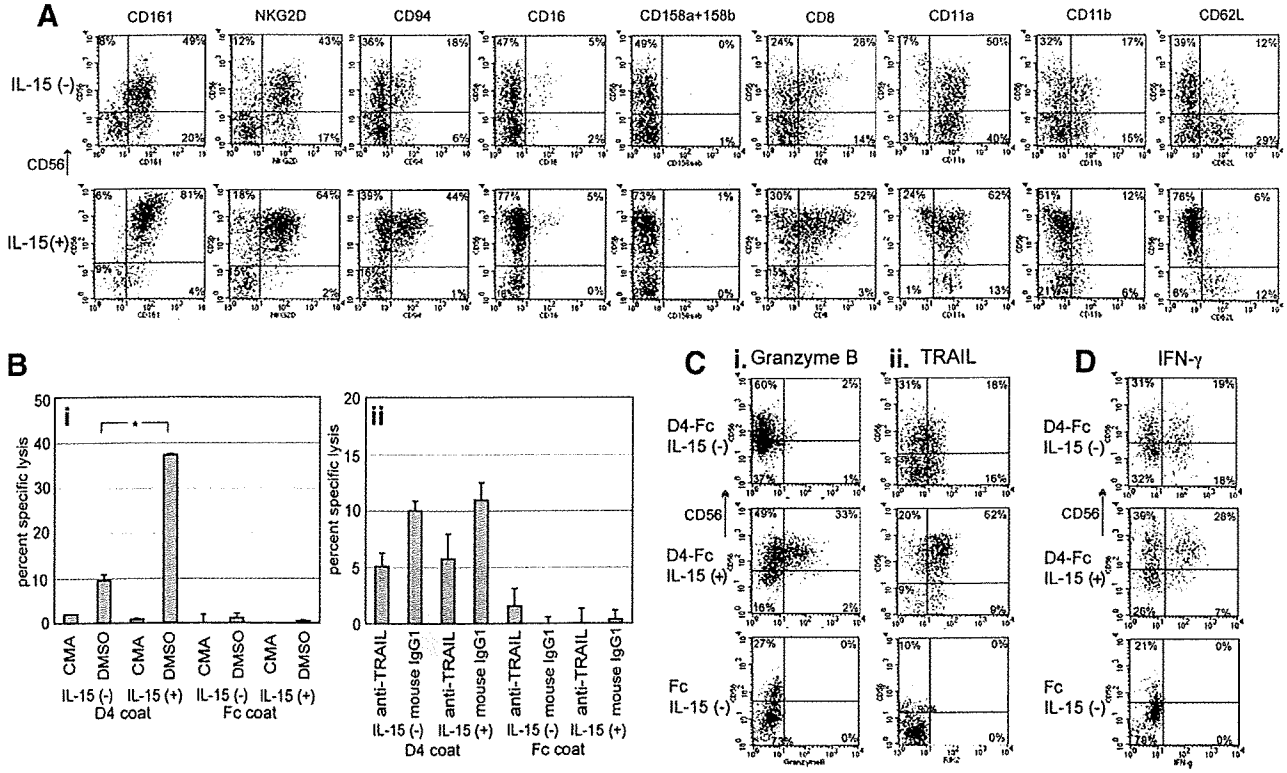

FIGURE 5. Phenotypic and functional differences between cells cultured in IL-15-containing and IL-15-free medium on Delta4-Fc-coated plates. *A*, Representative dot plots illustrating CD56 vs indicated Ags of cells cultured for 3 wk from CB CD34$^+$ cells in IL-15-containing or IL-15-free medium on Delta4-Fc-coated 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