Funktionelle Quantifizierung humaner hämatopoietischer Stammzellen *in vitro*

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vorgelegt von
Dr. med. Michael Punzel

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1. Einleitung

Die hämatopoietischen oder Blutstammzellen des Knochenmarks (HSZ) sind auf Grund ihrer guten Zugänglichkeit die am besten untersuchten Stammzellen des Menschen. Diese Zellen zeichnen sich durch ihre duale Fähigkeit zur langfristigen Selbsterneuerung und gleichzeitigen Differenzierung in ihre genetisch vorprogrammierten Entwicklungsrichtungen aus. Sie unterscheiden sich damit von den gewebsspezifischen Progenitorzellen, die sich nur noch begrenzt vermehren oder differenzieren können. Erstmals zu Beginn der sechziger Jahre des 20. Jahrhunderts konnten Blutstammzellen anhand ihrer klonalen Proliferationsfähigkeit in letal bestrahlten Mäusen identifiziert werden (1,2). Kurze Zeit später wurde berichtet, dass diese Progenitoren mit der Fähigkeit zur Bildung von klonalen Differenzierungskolonien (Colony Forming Cells, CFC) in der Lage sind, in sekundären Empfängertieren alle Blutzellreihen zu regenerieren. Deshalb wurde damals auch angenommen, dass diese Zellen den blutbildenden Stammzellen entsprechen (3). Bereits im Jahre 1968 wurde dann auf Grundlage dieser Erkenntnisse die erste erfolgreiche allogene Knochenmarktransplantation bei einem Kind mit schwerem kombiniertem Immundefekt (SCID) durchgeführt (4).

Große Anstrengungen sind seit dieser Zeit unternommen worden, um menschliche HSZ zu charakterisieren und anhand bestimmter phänotypischer Merkmale zu isolieren. Trotz weitgehender Identifizierung des Blutstammzellphänotyps in der Maus (5-7), gibt es bis zum heutigen Tag keine zuverlässigen Oberflächenmerkmale, die es erlauben, menschliche HSZ zu isolieren. Mit der Entwicklung von monoklonalen Antikörpern wurde das Oberflächenantigen CD34 als einer der wichtigsten Marker zur Anreicherung von humanen HSZ beschrieben (8-11). Die CD34+ Population ist jedoch in sich sehr heterogen und enthält neben sehr wenigen Stammzellen überwiegend liniendeterminierte Progenitoren. Da im humanen System die Expression von Oberflächenmarkern nicht systematisch mit funktionellen Eigenschaften korreliert, ist es bis heute nicht möglich, humane HSZ allein phänotypisch zu beschreiben (12-14).

Im Tierexperiment können Blutstammzellen anhand ihrer Fähigkeit zur Regeneration der Blutbildung in Transplantationsexperimenten analysiert und quantifiziert werden (5-7). Da solche Experimente im humanen System nicht möglich sind, wurden im Labor sogenannte *in vitro* Modellsysteme entwickelt, um die Eigenschaften primitiver blutbildender Zellen direkt zu untersuchen. Ausgehend von den initialen Beobachtungen um die koloniebildenden Vorläuferzellen (CFC), die in der Lage sind, mehr als 50 Zellen pro

Kolonie zu generieren, versuchte man in den achtziger Jahren dieses Modellsystem weiter zu entwickeln und den CFC hierarchisch vorausgehende Progenitoren zu definieren. Dabei wurde zunächst eine Zelle beschrieben, die als sogenannte „High proliferative potential-CFC“ (HPP-CFC) zwar ein etwas höheres Proliferationspotenzial besitzt ($5x10^4$ Zellen/Kolonie), aber nur unwesentlich unreifer als die CFC ist (15,16).

Wesentlich primitivere Zellen als die CFC wurden durch die Entwicklung der sogenannten Knochenmarklangzeitkulturen entdeckt. Diese Zellen sind nach mindestens 5 Wochen *in vitro* Kultur auf einer adhärenten Schicht von Knochenmarkstromazellen noch in der Lage, sekundäre CFC zu generieren. Diese den CFC hierarchisch übergeordneten myeloisch determinierten Progenitoren wurden als sogenannte „Long-term Culture Initiating Cells“ oder LTC-IC bezeichnet (17,18). Den LTC-IC eng verwandt sind Vorläuferzellen die sogenannte pflastersteinartige Proliferationsinseln bilden und deswegen auch als „Cobblestone Area Forming Cells“ oder CAFC bezeichnet wurden (19-21). Auf der Suche nach unreiferen und den Stammzellen noch enger verwandten Zellen wurde der LTC-IC-Test auf mehr als 60 Tage erweitert und sogenannte „extended“ LTC-IC (E-LTCIC) beschrieben (22).

Da sämtliche LTC-IC nur hinsichtlich ihrer myeloischen Differenzierungsfähigkeit in erythrozytäre und granulozytäre Sekundärkolonien getestet werden können, wurden auch lymphatische Langzeitkulturen entwickelt. Die unreifen lymphatischen Vorläuferzellen, die in diesen Testsystemen untersucht werden, sind nach 5 bis 7 Wochen Langzeitkultur noch in der Lage, multiple und funktionell reife NK- oder B-Zellen zu generieren. Je nach hämatologischer Differenzierung wurden diese Zellen als „Natural Killer Cell Initiating Cells“ (NK-IC) oder auch als „B-Cell Initiating Cells“ (B-IC) bezeichnet (23-26).

Bis heute ist es unklar, welche Übereinstimmung es zwischen Stammzellen und den in Langzeitkulturen nachgewiesenen Vorläuferzellen gibt. Im murinen System konnte mit Hilfe von Transplantationsexperimenten eine enge numerische Beziehung zwischen Stammzellen und den LTC-IC gefunden werden (27); die Übertragung auf das humane System erwies sich jedoch als problematisch: In unmanipuliertem Knochenmark oder Nabelschnurblut korreliert zwar die Anzahl von CD34+ Zellen, CFC oder LTC-IC-Progenitoren mit dem Stammzellgehalt und kann so für die Berechnung der Zelldosis eingesetzt werden. Nach *in vitro* Manipulation ist jedoch dieser numerische Zusammenhang verloren gegangen, da eine Expansion des Phänotyps oder bestimmter

Progenitorpopulationen keinen Rückschluss auf die Anzahl der noch vorhandenen Stammzellen erlaubt (12-14,28).

Als Alternative zu den *in vitro* Progenitorassays wurden Transplantationssysteme entwickelt, in denen menschliche Blutstammzellen durch ihre Fähigkeit zur Regeneration des blutbildenden Systems in immunsupprimierten Empfängertieren nachgewiesen werden können. Als wichtigstes xenogenes *in vivo* System haben sich dabei die NOD-SCID-Mäuse etabliert, die durch multiple Defekte des zellulären und humoralen Immunsystems gute Vorraussetzungen für das Anwachsen menschlicher Zellen bieten und inzwischen auch standardmäßig zur Bestimmung humaner Stammzellen verwendet werden. Aber auch andere Transplantationssysteme wie die *beige-nude*-Maus oder sogenannte *in utero* Transplantationen in fötalen Schafen haben sich zur Bestimmung menschlicher HSZ durchgesetzt (29-32).

Es ist unstrittig, dass Blutstammzellen für die Etablierung einer humanen Hämatopoiese in diesen Tieren verantwortlich sind. Allerdings ist es noch völlig unklar, wie viele mitübertragene liniendeterminierte Progenitoren ebenfalls zur menschlichen Blutbildung beitragen, wenn diese Tiere generell nach 6 bis 8 Wochen analysiert werden (33). Auch bestehen durch das nicht-humane Umgebungsmilieu der Untersuchungstiere Barrieren für das Anwachsen der menschlichen Zellen, was wiederum große Zellzahlen für die Transplantationsexperimente voraussetzt (33,34). In Primatenmodellen konnte erst kürzlich anhand von Gentransferexperimenten gezeigt werden, dass insbesondere das NOD-SCID-Maus-Modell die Anzahl menschlicher Stammzellen deutlich überbewertet (35). Aus diesen Gründen ist es noch völlig unklar, in welchem Maße diese *in vivo* Modellsysteme wirklich menschliche HSZ bestimmen.

2. Eigene Untersuchungen

Die zunehmende Entwicklung gentherapeutischer Ansätze für die Klinik sowie die Bemühungen, humane Stammzellen für klinische Anwendungen zu expandieren, machten Testsysteme notwendig, in denen stammzelläquivalente humane Zellen bestimmt werden können. Um ein solches Testsystem zu etablieren, mussten Selbsterneuerung und Differenzierung in verschiedene Linien der Hämatopoiese auf Einzelzellebene nachgewiesen werden.

2.1. Entwicklung eines *in vitro* Kultursystems zur Bestimmung von primitiven, multipotenten humanen hämatopoietischen Vorläuferzellen

2.1.1. Identifizierung eines optimalen Stromas zur Unterstützung primitiver hämatopoietischer Vorläuferzellen

Erstes Ziel der Arbeit war es, zunächst ein *in vitro* Kultursystem zu etablieren, dass reproduzierbar die Zahl von humanen LTC-IC und NK-IC in gegebenen Populationen quantifizieren kann. Diese Zellkulturen zur Identifizierung von primitiven hämatopoietischen Vorläuferzellen sind stark abhängig von einer Kokultur mit Knochenmarksstroma. Primäres Stroma von gesunden Spendern steht allerdings routinemäßig nicht immer zur Verfügung und liefert auf Grund der unterschiedlichen individuellen Qualität stark variierende Ergebnisse für die LTC-IC-Bestimmung (36,37). Deshalb wurde primäres Stroma mehr und mehr durch klonierte Zelllinien ersetzt, um vergleichbare und reproduzierbare Ergebnisse zu erzielen. Allerdings erschwert die Vielfalt dieser Zelllinien die Vergleichbarkeit der Ergebnisse zwischen unterschiedlichen Laboratorien (21,36-38).

Für die Etablierung eines humanen Stammzellassays erschien eine Reihe von neu etablierten murinen Zelllinien interessant, die im Maussystem erstmals in der Lage waren, transplantierbare Stammzellen über mehr als 7 Wochen *in vitro* zu erhalten und sogar zu expandieren (39-41). Wir testeten daraufhin 5 dieser Zelllinien auf die Unterstützung für humane LTC-IC und NK-IC. Eine dieser Zelllinien (AFT024), die aus fötalen Leberzellen der Maus etabliert wurde (40,41), war im Gegensatz zu primärem Stroma erstmals in der Lage, sowohl primitive myeloische LTC-IC über mehr als 5 Wochen in Kultur zu erhalten als auch lymphatische NK-IC aus CD34+/HLA-DR-/CD2-/CD7- Knochenmarkszellen zu generieren und in einem gemeinsamen myeloisch-lymphatischen Kultursystem zu expandieren (42,43).

Da die Fähigkeit der adhärenten Stromaschicht zur Unterstützung der hämatopoietischen Zellen entscheidend ist, war es von großem Interesse zu untersuchen, ob relative Änderungen der Progenitorfrequenzen vor und nach *in vitro* Manipulation nur auf Grund verschiedener Stromazellschichten unterschiedlich interpretiert werden können. Hier konnten wir erstmals zeigen, dass sowohl die absoluten LTC-IC-Frequenzen als auch relative Veränderungen nach Zellkultur auf verschiedenen Stromazellen signifikant unterschiedlich ausgezählt und damit je nach Stromazelllinie über- oder unterbewertet

werden (42). Diese Beobachtung hatte weitreichende Bedeutung für die *in vitro* Kultivierung, da z.B. die humanen HSZ nach Expansionskulturen oder Gentransferexperimenten über diese Progenitorzahlen determiniert werden.

2.1.2. Charakterisierung der Wechselwirkung zwischen primitiven hämatopoietischen und stromalen Zellen

Die unterschiedlichen Ergebnisse der Langzeitkultivierung primitiver humaner HSZ auf verschiedenen Stromazelllinien ist vermutlich durch eine vielfältige Wechselwirkung zwischen den hämatopoietischen Zellen und dem Stroma begründet. Direkter Kontakt der hämatopoietischen Zellen mit Komponenten des Stromas führt zu einer Hemmung der Zellzyklusprogression, was u.a. durch eine Integrin-vermittelte intrazelluläre Steigerung des Zellzyklusinhibitors p^{27kip1} erklärt werden kann (44). Die adhäsionsvermittelte Hochregulation dieses Inhibitors wiederum ist verantwortlich für das verminderte Überleben von primitiven hämatopoetischen Progenitoren bei direktem Stroma-Kontakt (45). Aus diesem Grund konnten in den bisher bekannten *in vitro* Langzeitkultursystemen humane Vorläuferzellen besser konserviert werden, wenn der direkte Kontakt mit dem Stroma durch semipermeable Membranen verhindert wurde (46). Überraschend war jedoch, dass die überdurchschnittliche Unterstützung der Zellinie AFT024 für eine multipotente humane Hämatopoiese besonders in direktem Kontakt mit dem Stroma zu beobachten war (43).

Da sich weder die sezernierten Zytokine noch die Makromoleküle der extrazellulären Matrix der Zelllinie AFT024 von denen der nichtsupportiven Zelllinien unterschieden (47), ergaben sich für die weitere Arbeit 2 Hypothesen: Erstens, die signifikante Verbesserung der *in vitro* Hämatopoiese auf der Zelllinie AFT024 könnte auf eine verminderte Adhäsion der hämatopoietischen Zellen und damit auf eine reduzierte Zell-Zell-Kontakthemmung zurückzuführen sein. Oder zweitens, AFT024-spezifische Faktoren könnten einer kontaktinduzierten Proliferationshemmung entgegenwirken.

Zur Beantwortung dieser Problematik führten wir eine Fixierung und metabolische Inaktivierung verschiedener Zelllinien durch, um isoliert Adhäsion und Kontakthemmung der HSZ durch das Stroma zu untersuchen. Im Ergebnis dieser Experimente konnten wir bei direktem Kontakt der HSZ mit der AFT024-Zellschicht weder einen Unterschied im Adhäsionsverhalten noch in der integrinvermittelten Proliferationshemmung im Vergleich zu anderen Linien festgestellen (43). Ursache waren vielmehr zelluläre Faktoren auf der

Oberfläche der AFT024-Zellen, die der Proliferationshemmung entgegenwirken und damit entweder Selbsterneuerungsteilungen ermöglichen und/oder die terminale Differenzierungsinduktion blockieren. Als Kandidaten wurden bereits mögliche Liganden des *Notch*-Rezeptors, wie *Dlk1* identifiziert (48). In eigenen Untersuchungen konnten zusätzlich auch Bestandteile der extrazellulären Matrix wie z.B. langkettige O-sulfatierte Heparansulfat-Glycosaminoglycane (GAGs) identifiziert werden, die spezifisch für AFT024 sind (43,47). Zwar konnte die isolierte Wirkung dieser Moleküle bisher noch nicht direkt an humanen HSZ getestet werden, wir zeigten aber, dass die supportiven Faktoren der Zelllinie AFT024 nicht sezerniert werden, sondern nur in direktem Zell-Zell-Kontakt wirksam sind (47). Allerdings können durch Zusatz von physiologischen Zytokinen die AFT024-Zellen angeregt werden, solche Faktoren in die Überstände zu sezernieren (47).

Mit der Zelllinie AFT024 war damit ein *in vitro* Kultursystem etabliert und untersucht worden, das sehr unreife Progenitoren mit myeloischem und lymphatischen Differenzierungspotenzial zur Proliferation anregt ohne terminale Differenzierungsschritte einzuleiten. Damit waren die Voraussetzungen geschaffen, um auf Einzelzellebene einen humanen Stammzellassay zu entwickeln.

2.2. Charakterisierung und Quantifizierung stammzelläquivalenter multipotenter hämatopoietischer Vorläuferzellen auf Einzelzellebene

2.2.1. Etablierung von Einzelzellassays für multipotente Progenitoren

Die etablierten Testsysteme für humane hämatopoietische Zellen werden in der Regel als Populationsassays in definierten Verdünnungsreihen, (sogenannte „Limiting Dilution Assays“ oder LDA) durchgeführt. Um jedoch Stammzellen entsprechend ihrer Definition zu beschreiben, sind Einzelzellanalysen notwendig, die für jede individuelle Zelle die Eigenschaften der Selbsterneuerung und multilinealen Differenzierungseigenschaften überprüft. Aus diesem Grund wurde zunächst versucht, optimale Bedingungen zur Einzelzelldifferenzierung in verschiedene myeloische und lymphatische Differenzierungs-richtungen zu etablieren.

Mit Hilfe eines Durchflusszytometers (FACS-Vantage®) mussten hochaufgereinigte, nicht-determinierte Knochenmarksvorläuferzellen einzeln in die Kultursysteme gegeben werden. Zu diesem Zweck wurde mittels monoklonaler Antikörper eine Population an Knochenmarksstammzell-Kandidaten isoliert, die nur CD34 exprimiert und negativ für

sämtliche myeloische und lymphatische Differenzierungsantigene (HLA-DR, CD2, 3, 4, 5, 7, 8, 10, 14, 15, 16, 19) ist. Nach durchflusszytometrischer Einzelablage dieser Zellen wurden zunächst Frequenzanalysen zur Differenzierungsfähigkeit in NK-Zellen, B-Zellen, dendritische, aber auch myeloid-erythroide Zellen durchgeführt (26). Eine Kombination aus den Zytokinen Interleukin-7 (IL-7), Flt-3Ligand (flt-3L), kit-Ligand (SCF) sowie die sequentielle Zugabe von Interleukin-3 (IL-3) und Interleukin-2 (IL-2) in Kokultur mit der Zelllinie AFT024 erwies sich als optimal, um die multipotente Differenzierungsfähigkeit der frühesten Vorläuferzellen über mehr als 5 Wochen *in vitro* zu erhalten und stabile und validierte Frequenzen dieser Zellen in unserer gewählten Population zu messen (26).

2.2.2. Quantitative Bestimmung sogenannter „Myeloid-Lymphoid-Initiating Cells“ (ML-IC)

Ausgehend von der Definition, dass nur die Zellen als Stammzellen zu charakterisieren sind, die sowohl die Fähigkeit zur Selbsterneuerung als auch zur Differenzierung in verschiedene Entwicklungsrichtungen besitzen, kam es in der experimentellen Planung darauf an, neben mindestens zwei Differenzierungslinien auch die sekundär-regenerative Funktion der Stammzellen eindeutig zu beweisen. Aus diesem Grund wurde das Assay-System so aufgebaut, dass jede initial sortierte Einzelzelle auf ihre Fähigkeit untersucht wurde, sowohl mindestens 2 LTC-IC als auch 2 NK-IC nach 4 Wochen Langzeitkultur zu generieren. Das bedeutete für das experimentelle Design, dass nach 4 Wochen Primärkultur weitere 7 Wochen sekundäre Differenzierungskulturen folgen mussten. Erst dann war eine Aussage möglich, ob die initiale Knochenmarkszelle in der Lage war, multiple und sekundär-regenerative Nachkommen verschiedener Differenzierungs-richtungen zu generieren und definitionsgemäß als „stammzelläquivalent“ zu gelten (49). Wir nannten solche Zellen „Myeloid-Lymphoid-Initiating Cells“ (ML-IC) und konnten in mehreren Versuchen zeigen, dass diese Zellen in nur sehr geringer Frequenz in verschiedenen Populationen von Knochenmark und Nabelschnurblut vorhanden sind (49,50). Im Knochenmark ermittelten wir eine Frequenz von 0,8% der sortierten CD34+/HLA-DR-/Lin- Population was ca. 4 ML-IC pro $1x10^5$ CD34+ Zellen entspricht (49). Im Nabelschnurblut ermittelten wir eine Frequenz von ca. 25 ML-IC pro $1x10^5$ CD34+ Zellen (50). Diese Zahlen sind vergleichbar mit der Anzahl der vermuteten HSZ aus tierexperimentellen Ansätzen (5-7,34). Andere Arbeitsgruppen konnten inzwischen unabhängig von uns bestätigen, dass ML-IC als sogenannte „stammzelläquivalente“ Progenitoren eine neue Qualität der *in vitro* Diagnostik darstellen und dass damit erstmals

quantitative Analysen auf individueller Stammzellebene im humanen blutbildenden System möglich sind (51,52).

2.3. Korrelation zwischen Zellteilungsverhalten und funktionellen Eigenschaften

Mit dem ML-IC-Assay ist es zwar möglich humane HSZ-äquivalente Zellen zu quantifizieren, aber diese Methode ist wie alle anderen Langzeitkulturen nur retrospektiv. Erst nach einer *in vitro* Kultivierung von mehr als 11 Wochen ist es möglich, eine Aussage zu treffen. Aus diesem Grund suchten wir nach einer Korrelation von funktionellen Eigenschaften der HSZ mit Parametern, die prospektiv eine Aussage über Stamm- oder Progenitorzellfunktion ermöglichen.

2.3.1. Quantitative Korrelation zwischen initialer Zellteilung und funktionellem Zellschicksal

Es ist allgemein bekannt, dass Stammzellen sich im Gegensatz zu linienspezifischen Progenitoren in relativer Zellteilungsruhe befinden. Aus diesem Grund untersuchten wir die Korrelation von initialem Zellteilungsverhalten jeder sortierten Einzelzelle mit ihren späteren funktionellen Eigenschaften. Mit Hilfe einer sequenziellen Mikroskopiertechnik, die Aufnahmen der Einzelzellen aller 12 bis 24 Stunden gewährleistet, untersuchten und dokumentierten wir die individuelle Teilungsgeschichte jeder einzelnen Zelle in einer 10tägigen Suspensionskultur. Anschließend wurden die Zellen zur Testung der funktionellen Eigenschaften (CFC, ML-IC) in Sekundärkulturen überführt, so dass es möglich war, für jede Einzelzelle funktionelle Eigenschaften und Zellteilungsverlauf direkt zuzuordnen.

Durch diesen experimentellen Ansatz waren wir in der Lage, erstmals auf Einzelzellebene direkte Unterschiede im initialen Teilungsverhalten zwischen koloniebildenden Progenitoren und stammzelläquivalenten ML-IC aufzuzeigen (50). Unabhängig von den *ex vivo* Kultivationsbedingungen teilen sich die bereits liniendeterminierten CFC in den ersten 48 Stunden nach Kulturbeginn mit hoher Proliferationsaktivität. Die Zellteilungen bei CFC sind zu 80% symmetrisch, d.h. aus jeder Tochterzelle der 1. Generation entwickeln sich wieder 2 Tochterzellen und so weiter. Als weiteres Charakteristikum der determinierten CFC wurde die schnelle Abfolge der initialen Teilungsabläufe erkannt. Über 90% der koloniebildenden Zellen durchliefen in den 10 Tagen der Zellbeobachtungsperiode

mindestens 3 mal den Zellzyklus. Im Gegensatz dazu rekrutierten sich die stammzelläquivalenten ML-IC aus einer völlig differenten Zellpopulation (50). CD34+/CD38-Zellen mit ML-IC-Kapazität zeigten, wie es bei Stammzellen allgemein bekannt ist, nur eine sehr geringe Teilungsaktivität: Je nach Kulturbedingung blieben mindestens 25% der ML-IC über die gesamten 10 Tage in Teilungsruhe und teilten sich, wenn überhaupt nur maximal zweimal. Im Gegensatz zu den CFC teilen sich die ML-IC zu ca. 90% asymmetrisch, d.h. eine der beiden Tochterzellen der 1. Generation bleibt in Teilungsruhe, während die andere weiter proliferiert (50).

Für das humane System wurde mit diesen Arbeiten erstmals auf Einzelzellebene bestätigt, dass sich unter identischen Bedingungen primitive ML-IC von liniendeterminierten CFC signifikant in Proliferationskinetik und Asymmetrie des initialen Zellteilungsverhaltens unterscheiden (50). Durch die klonale Analyse auf Einzelzellebene konnte auch gezeigt werden, dass primitive Vorläuferzellen wie z.B. ML-IC neben ihrer „Stammzellfunktion" keine zusätzlichen Eigenschaften hierarchisch untergeordneter Progenitoren wie z.B. die Fähigkeit zur Koloniebildung (CFC) besitzen, sondern dass die jeweiligen funktionellen Eigenschaften streng abgrenzbar immer aus unterschiedlichen Einzelzellen hervorgehen (50).

2.3.2. Einfluss extrinsischer Faktoren auf das initiale Zellteilungsverhalten

Aus der strengen Korrelation zwischen dem initialen Zellteilungsverhalten und der funktionellen Kapazität der Zellen ergab sich die Frage, ob die intrinsisch gesteuerten Zellteilungsmechanismen auch durch externe Faktoren beeinflussbar sind. Aus den klinischen Erfahrungen wissen wir, dass hämatopoietische Zellen sehr schnell auf externe Reize reagieren. Bei einer bakteriellen Infektion z.B. müssen sehr schnell linienspezifische reife myeloische Zellen (z.B. Granulozyten) aus den entsprechenden Progenitoren gebildet werden, ohne dass es dabei zu einem Stammzellverlust kommt. Andererseits zeigt uns die Praxis nach einer Blutstammzelltransplantation, dass bei einem normalen Anteil von ca. 1% CD34+ Zellen eine geringe Anzahl an transplantierten mononukleären Zellen ($2x10^8$ pro kg Körpergewicht) ausreicht, um bei einem myeloablativ konditionierten Patienten innerhalb von 14 Tagen eine vollständige hämatologische Rekonstitution zu erzielen. Aus diesem Grund müssen sowohl Expansions- als auch Differenzierungsteilungen auf Stammzellebene ablaufen.

Für mögliche *ex vivo* Manipulationen wie z.B. den retroviralen Gentransfer oder eine Transplantat-Expansion ist es von großer praktischer Bedeutung, ob und wie diese Regulationsmechanismen beeinflussbar sind. Zur Beantwortung dieser Frage kultivierten wir CD34+/CD38- Zellen mit standardisierten Zytokincocktails, die entweder die bereits determinierten Progenitoren (CFC) stimulieren und differenzieren (53) oder schon sehr „früh“ auf Stammzellniveau wirksam sind (47).

Wir konnten zeigen, dass sowohl Stamm- als auch Progenitorzell-Kandidaten unabhängig von der Stimulierung ein einheitliches Proliferationsverhalten zeigen, wenn sie isoliert und unabhängig von ihrem Umgebungsmilieu stimuliert werden: Die primitiven ML-IC rekrutierten sich zu 100% aus den nicht oder nur langsam proliferierenden Zellen, die als zusätzliches Charakteristikum noch eine signifikante Teilungs- und Proliferationsasymmetrie der Tochterzellen zeigten (50). Die frühwirksamen Zytokine bewirkten bei den sich nicht teilenden ML-IC den Eintritt in den Zellzyklus für maximal 2 Proliferationsschritte, änderten aber nichts am asymmetrischen Zellteilungsmuster. Die determinierten Progenitoren (CFC) teilten sich im Gegensatz zu den ML-IC auch unabhängig vom Zytokinmileu sehr rasch und überwiegend symmetrisch (50). Damit konnten wir zeigen, dass die Proliferations- und Zellteilungsprogramme der hämatopoietischen Stamm- und Progenitorzellen intrinsisch determiniert sind und durch lösliche Stimulatoren allein nicht oder nur unwesentlich beeinflusst werden können. Auch blieb die Zellteilungsasymmetrie der CD34+/CD38- Gesamtpopulation unbeeinflusst und lag bei ca. 20%, was frühere Ergebnisse anderer Arbeitsgruppen bestätigte (53).

Um den Einfluss des Umgebungsmilieus auf die initiale Zellteilung und deren funktionelle Auswirkungen zu testen, untersuchten wir in abschliessenden Arbeiten das Proliferationsverhalten von CD34+/CD38- Zellen, wenn sie bereits initial mit dem stammzellunterstützenden AFT024-Stroma kokultiviert werden (54). Dabei konnten wir zeigen, dass bei primärem Kontakt mit AFT024 mehr CD34+/CD38- Zellen als ML-IC identifiziert werden können als im Vergleich zu Suspensionskulturen ohne Stroma. Das bedeutet, dass durch das stammzellsupportive Umgebungsmilieu der AFT024-Zelllinie die primitivsten hämatopoietischen Zellen aus der tiefen G_0-Phase des Zellzyklus in die Proliferation rekrutiert werden. Gleichzeitig erhöhte sich sowohl die Anzahl der Zellzyklusdurchläufe für sich bereits teilende ML-IC als auch die Anzahl der asymmetrischen Zellteilungen um 10% (54). Diese Ergebnisse bestätigten die Hypothese, dass die Zellteilungs- und Differenzierungsvorgänge der HSZ überwiegend intrinsisch determiniert sind und in Suspensionskulturen durch Zytokine oder Adhäsionsmoleküle

nicht beeinflusst werden können. Erst wenn die Zellen in einem hämatopoietischen Umgebungsmilieu adhärent kultiviert werden, lassen sich extern induzierbare Veränderungen der Funktionalität und des Zellteilungsverhaltens nachweisen. Die intrinsische Kontrolle der überwiegend asymmetrischen Zellteilungen der ML-IC bestätigt auch das stark limitierte symmetrische Expansionspotenzial von Blutstammzellen *ex vivo*.

3. Zusammenfassung und Schlussfolgerung

Die Etablierung des hier dargestellten Langzeitkultursystems auf Einzelzellebene, welches die humane Blutzellbildung von der multipotenten Stammzelle bis hin zu funktionell reifen myeloischen und lymphatischen Zellen nachvollzieht, erlaubte erstmals die Charakterisierung und Quantifizierung von blutbildenden Stammzellen des Menschen *in vitro*. Mit dem Nachweis der Fähigkeit zur sekundären Regeneration einer multipotenten Hämatopoiese aus mindestens 2 individuellen Tochterzellen wurde eine qualitativ neue, humane hämatopoietische Vorläuferzelle beschrieben, die per Definition den blutbildenden Stammzellen äquivalent ist und als „Myeloid-Lymphoid-Initiating-Cell“ (ML-IC) bezeichnet wurde. Voraussetzung für die notwendige Langzeitkultur über mehr als 10 Wochen ist die Kokultivierung mit der Stromazelllinie AFT024. In ersten Ansätzen wurde die Spezifität der proliferativen Interaktionen mit den hämatopoietischen Zellen untersucht und beschrieben.

Die Quantifizierung primitivster hämatopoietischer Vorläuferzellen auf Einzelzellebene eröffnet die Möglichkeit der direkten Validierung bereits bestehender xenogener Transplantationssysteme zur Messung von humanen HSZ. Weiterhin kann man mit Hilfe dieses Kultursystemes spezielle *ex vivo* Bedingungen zur Differenzierungsinduktion bzw. Selbsterneuerungsteilung von HSZ in Abhängigkeit einzelner Zellzyklusschritte untersuchen. Die Ergebnisse dieser Experimente können für retrovirale Therapieansätze von erheblicher Bedeutung sein, da es bisher nicht möglich war, einzelne Proliferationsschritte in Abhängigkeit der Folgen auf das „Stammzellschicksal“ zu untersuchen. Die eigenen weiterführenden Arbeiten mit diesem System dienten der prospektiven Identifizierung von Stamm- und Progenitorzellkandidaten anhand funktioneller Zellteilungsparameter. Auf Einzelzellebene konnten dabei erstmals Charakteristika des initialen Teilungsverhaltens von liniendeterminierten Progenitoren und HSZ-äquivalenten Zellen dargestellt werden. Die hämatopoietischen Zellen unterliegen dabei einer sehr strengen intrinsischen Teilungskontrolle, die nur durch Determinanten

des Umgebungsmilieus beeinflusst werden kann. Primitive Zellen zeigen dabei eine ausgesprochene Preferenz zu asymmetrischen Zellteilungen bei nur sehr wenigen Zellzyklusschritten, wogegen sich die determinierten Progenitoren signifikant häufiger und vorwiegend symmetrisch teilen.

Die Ergebnisse zur asymmetrischen Zellteilung wurden inzwischen auch funktionell durch Mikromanipulationsstudien bestätigt, in denen gezeigt werden konnte, dass asymmetrisch teilende ML-IC ihr funktionelles Schicksal bereits während der initialen Zellteilung nur auf eine der beiden Tochterzellen übertragen (55). Diese funktionelle Teilungsasymmetrie hat hinsichtlich praktisch relevanter *in vitro* Kulturen wie z.B. die Kultur für retrovirale Transduktionen oder Expansionsansätze eine große praktische Bedeutung, da unter den gegebenen Bedingungen die Stammzellproliferation praktisch zu keiner Expansion führen kann.

Die Anwendung dieses *ex vivo* Systems in vergleichenden Untersuchungen mit etablierten Transplantationssystemen wird ergeben, ob und inwieweit ML-IC wirklich mit humanen hämatopoietischen Stammzellen übereinstimmen, denn die Fähigkeit zur Regeneration der humanen Hämatopoiese *in vivo* als wichtigste praktische Determinante der Stammzelldefinition wurde bisher nicht überprüft.

Die vielfältigen Anwendungen dieses experimentellen Systems für hämatopoietische Zellteilungs- und Differenzierungsstudien stellen im Zusammenhang mit der Quantifizierbarkeit der Stammzellen auf Einzelzellebene eine vielversprechende Alternative zu den xenogenen Transplantationsexperimenten dar, und eröffnen die Möglichkeit zu vergleichenden *ex vivo* und *in vitro* Versuchen auf Stammzellebene. Zusätzlich lassen sich auch mögliche Zellschicksalsdeterminanten menschlicher HSZ in diesem experimentellem System funktionell auf Einzelzellebene untersuchen.

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5. Anlagen

Sonderdrucke der folgenden Arbeiten:

Punzel M, Moore KA, Lemischka IR, Verfaillie CM. (1999) The type of stromal feeder used in limiting dilution assays influences frequency and maintenance assessment of human long-term culture initiating cells.
Leukemia. 13:92-7.

Punzel M, Gupta P, Roodell M, Mortari F, Verfaillie CM. (1999) Factor(s) secreted by AFT024 fetal liver cells following stimulation with human cytokines are important for human LTC-IC growth.
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The type of stromal feeder used in limiting dilution assays influences frequency and maintenance assessment of human long-term culture initiating cells

M Punzel[1], KA Moore[2], IR Lemischka[2] and CM Verfaillie[1]

[1]*Department of Medicine, Division of Hematology and Stem Cell Biology Program University of Minnesota, MN; and* [2]*Department of Molecular Biology, Princeton University, NJ, USA*

The goal of this study was to evaluate if differences in culture conditions used in long-term culture assays affect enumeration of LTC-IC in freshly sorted or *ex vivo* expanded $CD34^+/HLA\text{-}DR^{dim}/CD2^-/CD7^-$ ($34^+/Lin^-$) cells. The variables examined included different stromal feeders (murine bone marrow fibroblast cell line, M2-10B4 and murine fetal liver cell line, AFT024) and presence or absence of cytokines (MIP-1α + IL-3). The absolute LTC-IC frequency in $34^+/Lin^-$ cells measured in limiting dilution assays (LDA) on AFT024 ($4.45 \pm 0.69\%$) was significantly higher than in M2-10B4 ($1.45 \pm 0.20\%$) LDA. Addition of MIP-1α and IL-3 to AFT024 LDA increased the measured LTC-IC frequency to $6.8 \pm 0.9\%$. We also determined the fraction of LTC-IC that persisted after $34^+/Lin^-$ cells were cultured for 5 weeks by replating progeny in the three LDA readout systems. The measured LTC-IC maintenance was significantly lower when M2-10B4 LDA ($13.1 \pm 3.5\%$, $P < 0.05$) were used compared with AFT024 LDA ($36.6 \pm 5.5\%$) or AFT024 LDA supplemented with MIP-1α and IL-3 ($29.1 \pm 6.3\%$). Thus, the number of LTC-IC measured in freshly sorted 34^+ cells depends on the stromal feeder used in LDA assays. Furthermore, and most important, assessment of LTC-IC expansion or maintenance may vary significantly depending on the type of stromal feeder used to enumerate LTC-IC.

Keywords: LTC-IC-assessment; stromal feeder; M2-10B4; AFT024

Introduction

Hematopoietic stem cells (HSC) are cells that can both self renew and generate committed progenitors capable of differentiating into all types of blood cells.[1–4] Assessment of stem cell number, quality and characteristics in animals can be done by transplantation of limited numbers of cells in lethally irradiated recipients.[5–8] In humans, the number and characteristics of HSC can be assessed by transplantation in xenogenic animal models.[9–11] Another more widely used method of assessing primitive human progenitors is to enumerate them *in vitro* in long-term cultures.[12–14]

The assay most frequently used to characterize primitive progenitors is the long-term culture initiating cell assay (LTC-IC), first described by Sutherland *et al*.[13] In this assay, cells that can initiate and sustain hematopoiesis, ie generate secondary CFC for a minimum of 5 weeks in stroma-based cultures, are thought to represent primitive progenitors, termed LTC-IC.[13,14] The cobblestone-area forming cell assay (CAFC), first described by Ploemacher *et al*,[16] measures primitive cells using a more visual endpoint, ie production of 'cobblestones' for more than 5 weeks after initiation of the culture, rather then generation of secondary CFC. When cells are plated in limiting dilutions (LDA), enumeration of the absolute frequency of LTC-IC or CAFC is possible.[13,17,18] These assays can provide important information concerning the differentiation stage and proliferative capacity of hematopoietic progenitors.

However, the reported LTC-IC frequency in human bone marrow and cord blood populations varies significantly. For instance, the frequency of LTC-IC reported for steady-state bone marrow $CD34^+$ cells ranges between 0.1 and 8.6%.[19–21] For more enriched $CD34^+/HLA\text{-}DR^-$ cells, reported LTC-IC frequencies range from 0.7 to 2.1%.[21–23] LTC-IC frequencies in $CD34^+/CD38^-$ cells range from 2 to 8% in adult bone marrow[21,24] and from 0.2 to 56% in cordblood $CD34^+/CD38^-$ cells.[14,25,26] Differences in the stromal feeder used for LTC-IC LDA (primary marrow stroma,[14,20,21,25,27,28] SYS1,[29] NIH-3T3,[30] FHS-173-We,[30] M2-10B4,[19,26,28,30] FBMD-1[16] or MS5[31]) may contribute to the observed differences. In addition, differences in cell culture methods, such as frequency of medium changes, initial cell density or cytokines added to the culture medium, can influence the results.[27,28,32–34]

Since the competence of stromal feeders used to assess LTC-IC numbers may greatly influence the perceived number of LTC-IC in a given cell population, we assessed the ability of a recently cloned murine fetal liver cell line, AFT024, to support human LTC-IC and compared these results with results obtained using the murine fibroblast feeder, M2-10B4. We demonstrate that the type of stromal feeder used in LTC-IC LDA not only affects the determination of LTC-IC frequencies in uncultured cells but also significantly changes the perceived number of LTC-IC that is maintained in expansion cultures.

Materials and methods

Cell sources and cell preparation

Bone marrow was aspirated from the posterior iliac crest from healthy volunteer donors after informed consent using guidelines approved by the Committee on the Use of Human Subjects at the University of Minnesota. Mononuclear cells (MNC) were obtained by Ficoll–Hypaque (Sigma-Diagnostics, St Louis, MO, USA) centrifugation. MNCs were incubated with a biotinylated anti-CD34 monoclonal antibody (clone 12.8) for 30 min at 4°C. After washing, the cells were loaded on to an avidin-immunoaffinity column (Ceprate system; Cellpro, Bothell, WA, USA). The adsorbed $CD34^+$ cells were released by gentle agitation and washed twice with IMDM containing 20% fetal calf serum (FCS, Hyclone Laboratories, Logan, UT, USA).

Cell sorting

To obtain $CD34^+/HLA\text{-}DR^-/CD2^-/CD7^-$ ($34^+/Lin^-$) cells, $CD34^+$ enriched cells were incubated for 30 min at 4°C with

Correspondence: CM Verfaillie, University of Minnesota, Box 806 UMHC, 420 Delaware Street SE, Minneapolis, MN 55455, USA; Fax: 612 626 4074
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anti-CD34-biotin (clone 12.8; Cellpro), anti-HLA-DR-PE (BDIS), anti-CD2-FITC (BDIS) and anti-CD7-FITC (BDIS) followed by streptavidin-SA670 (GIBCO-BRL, Grand Island, NY, USA). Cells were sorted on a FACS Star-Plus flow cytometry system (Becton Dickinson, Immunocytometry Systems, San Jose, CA, USA) equipped with a CONSORT32 computer using isotype-matched controls (Figure 1).

Stromal feeders

The murine fibroblast cell line M2-10B4, (a kind gift from C Eaves, Terry Fox Laboratories, Vancouver, Canada) was maintained in RPMI 1640 (GIBCO-BRL) with 10% FCS. M2-10B4 supports human hematopoiesis in stroma-contact and stroma-non-contact cultures to the same extent as primary human bone marrow stromal feeders.[28,30] The murine fetal liver cell line, AFT024, was maintained at 33°C in DMEM (GIBCO-BRL) supplemented with 20% FCS, 50 μM 2-mercaptoethanol (2-ME) (Bio-Rad, Hercules, CA, USA). M2-10B4 or AFT024 cells were subcultured in 24- or 96-well plates (Costar, Cambridge, MA, USA), grown to confluency and then irradiated at 6000 rads (M2-10B4) or 2000 rads (AFT024) using a Mark 1 cesium irradiator (Shepard, Glendale, CA, USA). For AFT024, tissue culture dishes were precoated with 0.1% gelatin (Specialty Media, Lavalette, NJ, USA) before seeding the cells. After irradiation, all media were replaced by complete LTBMC-medium containing IMDM, 12.5% FCS, 12.5% horse serum (Stem Cell Technologies, Vancouver, Canada), 2 mmol/l L-glutamine (GIBCO), penicillin (1000 U/ml), streptomycin 100 U/ml (GIBCO) and 10^{-6} mmol/l hydrocortisone and cultures were maintained at 37°C.[35]

Expansion cultures

On day 0, 5000 freshly sorted $34^+/Lin^-$ cells were plated in collagen-coated transwell inserts (0.4-μm pore size) (Costar) placed above M2-10B4 stromal feeders.[23] Cultures were maintained for 5 weeks in LTBMC medium in a humidified atmosphere at 37°C and 5% CO_2. Cultures were fed at weekly intervals by removing half of the medium and replacing it with fresh complete LTBMC medium.

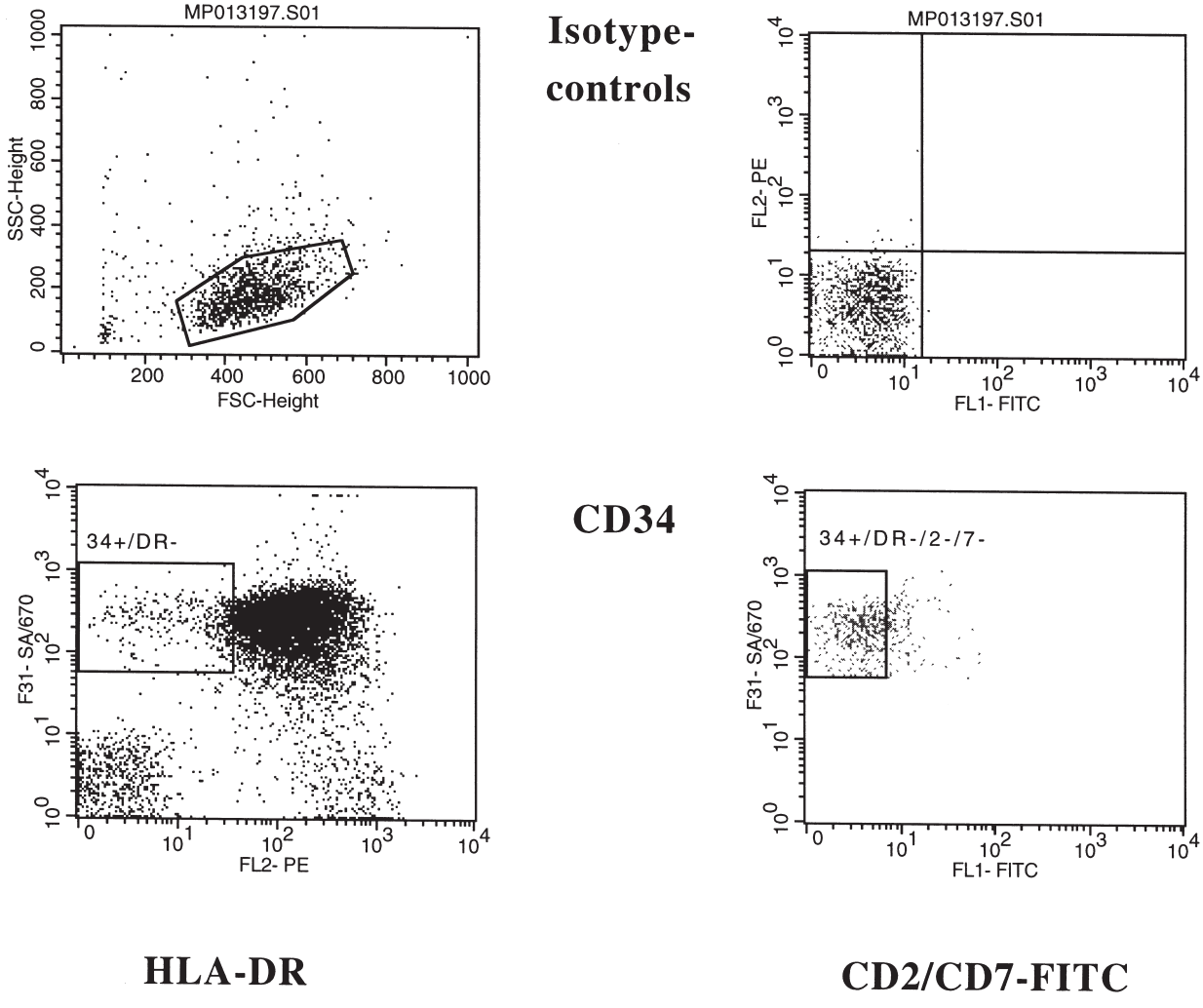

Figure 1 Column enriched $CD34^+$ cells were stained with MoAbs against $CD34^+$ (biotin-SA670), HLA-DR (PE), CD2 (FITC) and CD7 (FITC). Cells present in the lymphoid window (left upper panel) were selected based on expression of CD34, absence/low expression of HLA-DR (left lower panel) and absence of CD2 and CD7 (lower right panel). Isotype control stains are shown in the upper right panel.

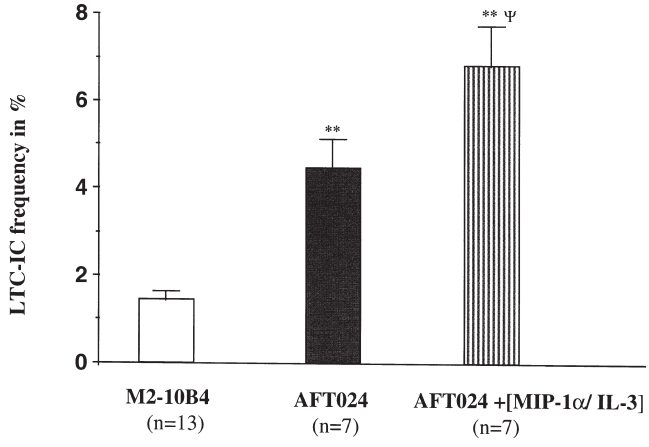

Figure 2 Assessment of LTC-IC frequency in freshly sorted $34^+/Lin^-$ cells depends on stromal feeders and culture medium used in LDAs. $34^+/Lin^-$ cells were plated in limiting dilutions on M2-10B4 feeders or on AFT024 feeders supplemented with or without MIP-1α (100 ng/ml) and IL-3 (5 ng/ml). After 5 weeks, LTBMC-media were removed and wells were overlaid with clonogenic methylcellulose medium. At day 12–16 wells were scored for CFC. $^{**}P < 0.01$ (LTC-IC frequency on AFT024 feeders *vs* M2-10B4 feeders); $^{\Psi}P < 0.05$ (LTC-IC frequency on AFT024 feeders *vs* AFT024 feeders supplemented with MIP-1α/IL-3).

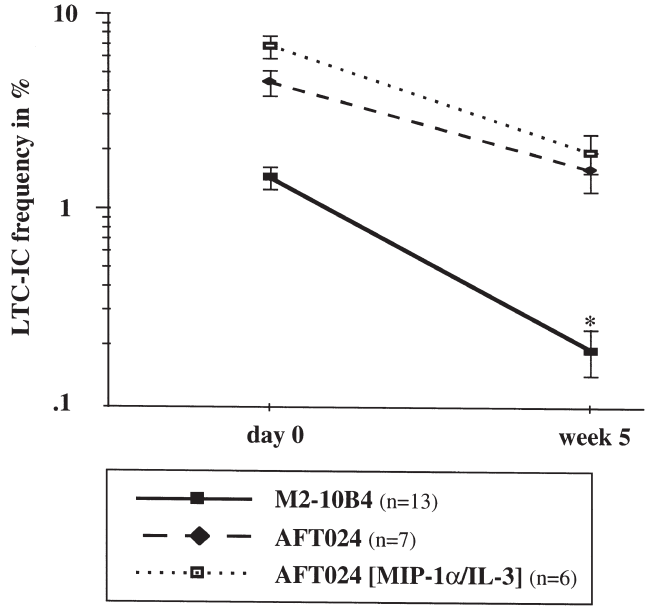

Figure 3 Assessment of LTC-IC maintenance depends on the type of feeder used in LDA readout assays. $34^+/Lin^-$ cells were cultured for 5 weeks in transwells above M2-10B4 feeders. The LTC-IC frequency was determined in three different LDA readout systems on day 0 and at week 5 (M2-10B4-LDA, AFT024-LDA with and without IL-3 and MIP-1α). Maintenance of LTC-IC was calculated as the absolute number of LTC-IC at week 5 divided by the absolute number at day 0 multiplied by 100. $^*P < 0.05$ (LTC-IC maintenance on AFT024 feeders *vs* M2-10B4 feeders).

Assessment of the absolute number of LTC-IC by limiting dilution assay (LDA)

On day 0, 5000 freshly sorted $34^+/Lin^-$ cells (22 replicates per concentration: 150, 50, 15, 5 cells/well) were plated on to irradiated confluent M2-10B4 or AFT024 feeders subcultured in 96-well plates (Costar) and maintained for 5 weeks in LTBMC medium with or without 100 ng/ml MIP-1α (R&D Systems, Minneapolis, MN, USA) and 5 ng/ml IL-3 (R&D Systems).[36] Likewise, week 5 progeny of $34^+/Lin^-$ cells was harvested from transwell inserts of M2-10B4 non-contact cultures and replated in M2-10B4 or AFT024-based LDA as described.[23] After 5 weeks, all LTBMC media were removed and replaced with clonogenic methylcellulose medium containing 1.12% methylcellulose, IMDM (GIBCO), 30% FCS (Hyclone), 3 IU/ml erythropoietin (Amgen, Thousand Oaks, CA, USA) and supernatant of the bladder carcinoma cell line 5637 (7.5%). Wells were scored for the presence or absence of secondary CFC between day 12 and day 16. The absolute frequency of LTC-IC present in the different cell populations was calculated as the reciprocal of the concentration of test cells that gives 37% negative cultures using Poisson statistics and the weighted mean method.[17,18] Maintenance of LTC-IC was determined by comparing the absolute frequency of LTC-IC on day 0 and at week 5.

Evaluation of CFC generated per LTC-IC

Two thousand $34^+/Lin^-$ cells were cultured on M2-10B4 feeders or AFT024 feeders in LTBMC medium with weekly media exchange. In some AFT024 cultures MIP-1α (100 ng/ml) and and IL-3 (5 ng/ml) were added to the LTBMC medium. After 5 weeks, cells were harvested and replated in clonogenic methylcellulose medium. The clonogenic cultures were assessed at days 14–16 for the presence of CFC as described.[37] The number of CFC per LTC-IC was calculated by dividing the total number of CFC/100 cells at week 5 in M2-10B4, AFT024 or AFT024/MIP-1α/IL-3 cultures by the absolute frequency of LTC-IC measured in M2-10B4, AFT024 or AFT024/MIP-1α/IL-3 LDA assays.

Statistics

Results of experimental points from different experiments were reported as the mean ± standard error of the mean (s.e.m.). Significance levels were determined by either paired or non-paired two-sided Student's *t*-test analysis as indicated.

Results

Assessment of the absolute frequency of LTC-IC in $34^+/Lin^-$ cells depends on the stromal feeder used in the LTC-IC-LDA system

Freshly isolated $34^+/Lin^-$ cells from normal bone marrow were plated in limiting dilutions on M2-10B4 or AFT024-feeders to assess the frequency of LTC-IC. Three-fold more $34^+/Lin^-$ cells generated secondary CFC in AFT024-LDA than in M2-10B4-based LDA (4.45 ± 0.69% *vs* 1.45 ± 0.20%; $P < 01$) (Figure 2). We have previously shown that addition of MIP-1α and IL-3 to long-term *ex vivo* expansion cultures results in complete maintenance of LTC-IC for at least 8 weeks, suggesting that this cytokine mixture provides support for *ex vivo* LTC-IC growth.[36] Therefore, we added MIP-1α (100 ng/ml) and IL-3 (5 ng/ml) weekly to AFT024-LDA assays and showed that the frequency of $34^+/Lin^-$ cells that can generate secondary CFC increased to 6.8 ± 0.9% (Figure 2). As shown in Table 1, the

Table 1 LTC-IC recruitment on M2-10B4 and AFT024 feeders

Stromal feeders used in LDAs to detect LTC-IC	*CFC frequency at week 5%*	*Absolute frequency of LTC-IC %*	*CFC/LTC-IC*
M2-10B4 ($n = 13$)	5.9 ± 1.7	1.5 ± 0.2	3.9
AFT024 ($n = 7$)	16.2 ± 2.2[b]	4.5 ± 0.7[b]	3.6[a]
AFT024 + (MIP-1α/IL-3) ($n = 7$)	21.1 ± 3.1[b]	6.8 ± 0.9[b]	3.1[a]

[a]CFC determined in non-paired experiments ($n = 5$).
[b]$P < 0.01$ for M2-10B4-feeders *vs* AFT0-24 feeders.

number of secondary CFC generated per single LTC-IC after culture in the three different conditions was not significantly different (3.9 CFC/LTC-IC on M2-10B4, 3.6 CFC/LTC-IC on AFT024 and 3.1 CFC/LTC-IC on AFT024/MIP-1α/IL-3).

The type of stromal feeder used in LTC-IC-limiting dilution assays affects assessment of LTC-IC maintenance/expansion

To investigate if the feeder used in LTC-IC LDA affects the assessment of maintenance/expansion of LTC-IC, we replated $34^+/Lin^-$ progeny cultured for 5 weeks in transwells above M2-10B4 feeders (non-contact cultures) in M2-10B4-LDA and AFT024-LDA supplemented with and without MIP-1α and IL-3. Using AFT024 feeders in the LDA readout assay, we show that $37.5 \pm 8.1\%$ of $34^+/Lin^-$ LTC-IC were maintained for 5 weeks in the absence of MIP-1α and IL-3 and $29.1 \pm 6.3\%$ of LTC-IC were maintained when LDAs were supplemented with MIP1-α and IL-3. Surprisingly, when M2-10B4-LDA were used to assess LTC-IC maintenance, significantly less LTC-IC ($13.1 \pm 3.4\%$, $P < 0.05$) were maintained (Figure 3).

Discussion

We demonstrate that the absolute frequency of LTC-IC measured in a given $CD34^+$ subpopulation depends on the type of stromal feeder used in the LDA as well as on the presence or absence of cytokines in the LDA assay. Using M2-10B4 feeders in the LDA assay, we detected an absolute frequency of $1.45 \pm 0.2\%$ LTC-IC in $34^+/Lin^-$ cells. This is similar to what we and others described previously using either M2-10B4 feeders or primary human bone marrow stromal feeders.[22,28,30] When AFT024 cells were used as feeder in the LDA assays, the measured LTC-IC frequency was $4.45 \pm 0.7\%$. It is possible that the increased number of LTC-IC measured in AFT024-LDA may be due to recruitment of more immature progenitors. However, since similar numbers of CFC are generated per LTC-IC on AFT024 and M2-10B4 feeders, the differentiation stage of LTC-IC that is allowed to grow in M2-10B4 or AFT024-LDA assays may be similar. Another possibility is that AFT024 feeders produce one or more factors that either prevent terminal differentiation or apoptosis of LTC-IC, or allow LTC-IC to proliferate. mRNA for more than 15 known cytokines can be found in AFT024 and this expression pattern does not differ significantly from that seen in less supportive feeders.[38,39] Moore *et al*[38–42] have recently shown that AFT024-cells express novel factors involved in hematopoietic stem cell regulation. One of the factors expressed by AFT024 cells is the delta-like (dlk) protein, pre-adipocyte factor-1 (pref-1).[38,43,44] When ectopically expressed in a hematopoietic non-supportive cell line, the dlk protein increases the *ex vivo* support of primitive murine hematopoietic progenitors.[38] Thus, dlk, produced by AFT024, but not M2-10B4 (unpublished observations) and/or other novel molecules expressed by AFT024 may block differentiation and/or apoptosis or may allow proliferation of primitive progenitors, resulting in the higher LTC-IC frequency measured in AFT024 LDA.[38,40–42]

We have reported that MIP-1α and IL-3 increases maintenance of LTC-IC when DR^- cells are cultured for 8 weeks in transwells above M2-10B4 feeders.[36] We show here that addition of MIP-1α and IL-3 to AFT024-LDAs further enhanced the measured LTC-IC frequency. This is consistent with reports from other investigators. Sutherland *et al*[22,28] have shown that the measured LTC-IC frequency can be increased by adding cytokines such as IL-3 and G-CSF to the culture medium or by plating progenitors on cytokine engineered feeders. Thus, our studies show that differences in the supportive feeders or addition of cytokines to the culture medium significantly influence the measured frequency of primitive progenitors in LTC-IC LDA assays.

Several groups are in the process of developing *ex vivo* culture systems to expand primitive and/or committed progenitors for transplant purposes. Likewise, short-term culture is needed for retroviral gene transfer. The availability of assays that can accurately measure the number of primitive and committed progenitors still present after *ex vivo* culture is of the utmost importance in assessing the effect of such *ex vivo* manipulations. We investigated if differences in the supportive capacity of feeders used in LDA readout assays influence our ability to assess maintenance or expansion of primitive hematopoietic progenitors after *ex vivo* long-term culture. The type of feeder layer used in LTC-IC LDA significantly affected the assessment of LTC-IC maintenance when $34^+/Lin^-$ cells were evaluated. The M2-10B4-LDA readout system underestimated the proportion of LTC-IC that persisted for 5 weeks in M2-10B4 stroma non-contact cultures by 50% when compared with AFT024 LDA with and without supplementation of MIP-1α and IL-3. This suggests that LTC-IC still present after 5 week long-term culture require certain stromal signals to proliferate provided by AFT024 feeders but not M2-10B4. If novel factors, such as dlk/pref-1, are responsible for the higher LTC-IC frequency measured in $34^+/Lin^-$ cells cultured *ex vivo* for 5 weeks remains to be determined.[42]

In conclusion, we illustrate here that enumeration of LTC-IC in a given cell population has to be interpreted cautiously. First, the feeder used in the LTC-IC LDA readout assays can greatly affect the determination of the absolute number of LTC-IC. Second, the culture medium used to maintain LTC-IC LDA affects the perceived LTC-IC frequency. Third, the ability of the feeder to support LTC-IC used in LDA can significantly affect the measured maintenance and/or expansion of LTC-IC. Differences in LTC-IC quantitation dependent on culture conditions rather than the progenitor population itself are of

concern when stroma-based LDAs are used to determine the efficacy of an *ex vivo* expansion system to maintain/expand primitive progenitors. Stromal feeder known to support maximally the growth of a given progenitor population should therefore be used to assess LTC-IC frequency/expansion.

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Factor(s) secreted by AFT024 fetal liver cells following stimulation with human cytokines are important for human LTC-IC growth

M Punzel[1], P Gupta[2], M Roodell[3], F Mortari[3] and CM Verfaillie[1]

[1]*Department of Medicine, Division of Hematology and Stem Cell Biology Program University of Minnesota;* [2]*Department of Veterans Affairs Medical Center;* [3]*R&D Systems, Biotech Division, Minneapolis, MN, USA*

Soluble factors produced by human marrow stroma or the murine marrow derived M2-10B4 cell line support *ex vivo* maintenance for 5–8 weeks of 50% of human long-term culture initiating cells (LTC-IC). As the AFT024 cell line supports LTC-IC cultured in contact conditions better than M2-10B4 feeders, we evaluated LTC-IC support in non-contact conditions above AFT024 feeders. We show that only 15% of LTC-IC were maintained for 5 weeks in AFT024 non-contact cultures ($n = 6$, $P < 0.05$). As AFT024-conditioned media added to M2-10B4 non-contact cultures did not inhibit LTC-IC maintenance, AFT024 cells do not secrete factors that inhibit LTC-IC growth. We next characterized heparan sulfate glycosaminoglycans (HS-GAGs) and cytokines produced by AFT024 cells, which are both required for LTC-IC maintenance in M2-10B4 non-contact cultures. The size and extent of O-sulfation of HS-GAGs in AFT024 and M2-10B4 conditioned medium were similar, indicating that absence of hematopoietic specific HS-GAGs is not responsible for the lack of hematopoietic in AFT024 non-contact cultures. Levels of 13 different cytokines secreted in AFT024- and M2-10B4-conditioned medium were similar. However, addition of human SCF, G-CSF, GM-CSF, LIF, MIP-1α and IL-6 in concentrations found in human marrow stroma-conditioned medium to AFT024 non-contact cultures increased LTC-IC-maintenance to 72% at 5 weeks. These cytokines improved LTC-IC maintenance in part through interaction with the progenitors and in part, through interaction with the AFT024 feeder. Thus, although LTC-IC maintenance is poor in AFT024 non-contact cultures, addition of human cytokines enhances LTC-IC maintenance in part through indirect effects on the AFT024 feeder. Characterization of known or novel growth factors secreted by AFT024 cells before and after cytokine stimulation may lead to the identification of cytokines that support growth of human hematopoietic stem cells.
Keywords: human LTC-IC; AFT024; stroma non-contact; human cytokines

Introduction

Under steady-state conditions hematopoiesis occurs in close contact with the bone marrow microenvironment. Proliferation and differentiation of hematopoietic stem cells (HSC) is regulated by soluble factors, as well as adhesive interactions between progenitors and the stroma. The hematopoietic process can be mimicked *in vitro*, by culturing progenitors on confluent layers of supportive stromal cells. A number of murine and human hematopoietic stromal cell lines have been described which support primitive human hematopoietic progenitors.[1–5] We and others have shown that primitive LTC-IC and primitive lymphoid progenitors can be maintained in stroma-based cultures even without contact with the stromal feeders.[6–11] Up to 50% of LTC-IC are maintained without exogenous cytokines in transwells above primary human bone marrow stroma or the murine fibroblast line M2-10B4, for 8 weeks.[3] Addition of IL-3 and MIP-1α increases this to 100%.[7] Likewise, primitive lymphoid progenitors are maintained in M2-10B4 non-contact cultures supplemented with MIP-1α and IL-3.[8] Although other cell lines, such as NIH3T3 and FHS-173-We, support LTC-IC in contact cultures equally well as M2-10B4 feeders, these two feeders poorly support LTC-IC in non-contact conditions.[3] We and others have shown that the murine fetal liver cell line, AFT024, supports human LTC-IC as well as lymphoid progenitors when cultured in contact with the feeder and this to a greater extent than primary stroma or M2-10B4.[12–14] The cell line also supports *ex vivo* maintenance of murine long-term competitive repopulating HSC for more than 7 weeks.[15] We now examine if AFT024 non-contact cultures would also support human LTC-IC.

We show that in contrast to M2-10B4-based cultures AFT024 non-contact cultures poorly support LTC-IC. This is not due to inhibitory factor(s) secreted by AFT024 feeders or lack of known cytokines or GAGs. Addition of human cytokines in concentrations found in marrow stroma-conditioned medium to AFT024 non-contact cultures significantly enhanced LTC-IC support, both through direct effects on hematopoietic progenitors and indirect effects on the AFT024 feeder.

Materials and methods

Cell sources

Bone marrow was aspirated from the posterior iliac crest from healthy volunteer donors after informed consent using guidelines approved by the Committee on the Use of Human Subjects at the University of Minnesota.

Cell preparation and cell sorting

Mononuclear cells were obtained by Ficoll–Hypaque (Sigma Diagnostics, St Louis, MO, USA) centrifugation. $CD34^+$ cells were enriched using CEPRATE columns (CellPro, Bothell, WA, USA). $CD34^+$ enriched cells were labeled with anti-CD34-PE (HPCA2, Becton-Dickinson Immunocytometry Systems (BDIS, San Jose, CA, USA) and anti-HLA-DR-FITC antibodies (BDIS). Cells were sorted on a FACS Star-Plus flow cytometry system (BDIS) equipped with a CONSORT32 computer using isotype-matched controls to obtain $CD34^+$/HLA-DR^- cells.[12]

Stromal feeders

The murine marrow derived cell line M2-10B4, (a kind gift from C Eaves, Terry Fox Laboratories, Vancouver, Canada) was maintained in RPMI1640 (Gibco-BRL, Grand Island, NY, USA) +10% fetal calf serum (FCS, Hyclone, Logan, UT, USA).

Correspondence: CM Verfaillie, Division of Hematology Oncology and Transplantation, Director, Stem Cell Biology Program, Box 806 UMHC, 420 Delaware Street SE, Minneapolis, MN 55455, USA; Fax: 612 626-4074
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The murine fetal liver cell line, AFT024 (a kind gift from IR Lemischka, Princeton University, Princeton, NJ, USA) was maintained in DMEM (Gibco) supplemented with 20% FCS, 50 μM 2-mercaptoethanol (BioRad, Hercules, CA, USA) and maintained at 33°C. M2-10B4 or AFT024 cells were subcultured in 24- or 96-well plates (Costar, Cambridge, MA, USA), grown to confluency and then irradiated at 6 Gy (M2-10B4) or at 2 Gy (AFT024). For AFT024, tissue culture dishes were precoated with 0.1% gelatin (Specialty Media, Lavalette, NJ, USA) before seeding the cells. After irradiation, all medium was replaced by LTBMC medium.

Culture media

Long-term culture medium (LTBMC medium): IMDM (Gibco) with 12.5% FCS, 12.5% horse serum (Terry Fox Laboratories, Vancouver, Canada), 2 mmol/l L-glutamine (Gibco), penicillin (1000 U/ml), streptomycin 100 U/ml (Gibco) and 10^{-6} mmol/l hydrocortisone.

AFT024- and M2-10B4-conditioned medium: AFT024 (M2-10B4) feeders were grown to confluency and irradiated as described.[12] After irradiation, all medium was replaced by LTBMC medium and incubated for 48 h. Conditioned medium was harvested and stored at –80°C until use.

Cytokine-conditioned AFT024 medium: AFT024 stroma was grown to confluency and irradiated as described.[12] After irradiation, all medium was replaced by RPMI 1640 (Gibco) medium with 20% FCS, 25 μM 2-ME, 1000 U/ml penicillin, 100 U/ml streptomycin (Gibco) and a combination of 10 pg/ml GM-CSF (Immunex, Seattle, WA, USA), 250 pg G-CSF (Amgen, Thousand Oaks, CA, USA), 200 pg SCF (R&D Systems, Minneapolis, MN, USA), 50 pg LIF (R&D), 200 pg MIP-1α (R&D), and 1 ng/ml IL-6 (a kind gift from Dr G Wong, Genetics Institute, Boston, MA, USA) and incubated for 48 h. Conditioned medium was harvested and stored at –80°C until use.

Long-term cultures

Non-contact cultures: Freshly sorted CD34$^+$/HLA-DR$^-$ cells were plated in collagen-coated transwell inserts (0.4-μm pore size) (Costar) placed above either M2-10B4 or AFT024 feeders. Cultures were maintained for 5 weeks with LTBMC medium in a humidified atmosphere at 37°C and 5% CO_2. Cultures were fed at weekly intervals by removing half of the medium and replacing it with fresh LTBMC medium. In some experiments M2-10B4 cultures were fed weekly with AFT024-conditioned medium. In some experiments the combination of six human cytokines at concentrations described above was added weekly to AFT024 non-contact cultures. In other experiments, a combination of five human cytokines was added omitting one of the six cytokines per experimental point.

Conditioned medium cultures: CD34$^+$/HLA-DR$^-$ cells were plated in bovine serum albumin (Sigma) coated 24-well plates. Cultures were fed every 48 h with AFT024-conditioned medium with AFT024 conditioned medium to which the six cytokine combination was added or with cytokine-conditioned AFT024 medium as described above.

Assessment of the absolute number of LTC-IC by limiting dilution assay (LDA)

On day 0, freshly sorted CD34$^+$/HLA-DR$^-$ cells were plated in four dilutions (22 replicates per concentration: 150, 50, 15, 5 cells/well) on irradiated confluent M2-10B4 feeders in 96-well plates and maintained for 5 weeks in LTBMC medium. Likewise, progeny of CD34$^+$/HLA-DR$^-$ cells cultured for 5 weeks were replated in different dilutions on M2-10B4 as done on day 0. Cultures were maintained for 5 weeks. LTBMC medium was removed and replaced with methylcellulose (1.12% final concentration, Fisher Scientific, Fair Lawn, NY, USA) containing medium supplemented with 3 IU/ml erythropoietin (Amgen, Thousand Oaks, CA, USA) and supernatant of the bladder carcinoma cell line 5637 (7.5%). Wells were scored for the presence or absence of secondary CFC between day 12 and day 16. The absolute number of LTC-IC present in the different cell populations was calculated as described.[6] Maintainance of LTC-IC was determined by comparing the absolute frequency of LTC-IC on day 0 and week 5.

Structural analysis of heparan sulfate glycosaminoglycans (HS-GAGs)

Purification of proteoglycans: Proteoglycans synthesized by irradiated M2-10B4 and AFT024 cells were labeled with 20 μCi/ml ^{3}H-glucosamine (to label the GAG backbone; DuPont NEN, Boston, MA, USA) and 50 μCi/ml $Na_2{}^{35}SO_4$ (to label the sulfate groups; ICN Biomedicals, Irvine, CA, USA) in LTBMC medium for 18–24 h. Proteoglycans in conditioned medium of the two cell lines were purified by anion exchange HPLC as previously described.[16,17]

Preparation of HS-GAGs: Purified proteoglycans were digested with chondroitinase ABC (cABC; Seikagaku America, Rockville, MD, USA) (which digests chondroitin and dermatan sulfate) and chromatographed on a Sephadex G-50 column. The material eluting at V_0 was treated with sodium hydroxide in the presence of sodium borohydride ($NaBH_4$), and free heparan sulfate chains released from the core protein were obtained by rechromatography on a Sephadex G-50 column.[17]

Estimation of size of HS-GAGs: The size of HS-GAGs was estimated by gel filtration chromatography on a Sepharose CL-6B column equilibrated in 4 M guanidine hydrochloride and 0.05 M sodium acetate, pH 5.8, as described earlier.[17] The approximate size of HS-GAGs was estimated by the method of Wasteson.[18]

Distribution of N-sulfation and O-sulfation in HS-GAGs: HS-GAGs from both cell lines was subjected to low pH nitrous acid (pH 1.5) deaminative cleavage of N-sulfated regions as described.[19] The digested oligosaccharides were resolved by gel filtration chromatography on a Sephadex G-25 column equilibrated in 0.2 M ammonium acetate, pH

7.0. The resulting oligosaccharides were analyzed as described in detail earlier.[17,20]

ELISA for murine cytokines in AFT024 and M2-10B4 supernatants

AFT024-conditioned medium, M2-10B4-conditioned medium, medium from AFT024 non-contact cultures supplemented with the combination of six human cytokines, and medium from AFT024 non-contact cultures supplemented with a combination of five human cytokines (omitting one cytokine per experimental point) were harvested and frozen at −80°C. The concentration of the murine cytokines IL-1β (sensitivity: 3 pg/ml), IL-3 (sensitivity: 2.5 pg/ml), IL-6 (sensitivity: 3.1 pg/ml), IL-10 (sensitivity: 4 pg/ml), IL-12p40 (sensitivity: 4 pg/ml), IL-12p70 (sensitivity: 2.5 pg/ml), GM-CSF (sensitivity: 1 pg/ml), MIP-1α (sensitivity: 1.5 pg/ml), MIP-1β (sensitivity: 1.5 pg/ml), MIP-2 (sensitivity: 1.5 pg/ml), TNFα (sensitivity: 5.1 pg/ml), TPO (sensitivity: 3 pg/ml) and SCF (sensitivity: 4 pg/ml) was determined using a quantitative enzyme immunoassay following the manufacturer's instructions (R&D Systems). Fifty μl of each medium was tested in duplicates, such that the resultant OD reading fell within the detection limits of each assay. The sensitivity of each assay is derived by adding two standard deviations to the mean optical reading value of replicate readings of the zero standard.

Statistics

Results of experimental points from different experiments were reported as the mean ± standart error of the mean (s.e.m.). Significance levels were determined by either paired or non-paired Student's *t*-test analysis as indicated.

Results

Human LTC-IC can be maintained when cultured in transwells above M2-10B4 feeders, but not NIH3T3 or FHS-173-We feeders.[3] More than 50% of human marrow or cord blood LTC-IC can be maintained when cultured in contact with AFT024 feeders.[11,14] As non-contact culture conditions or culture in conditioned medium may be more easily adapted to the clinical setting, we here tested if human marrow derived LTC-IC can be maintained in AFT024 non-contact conditions. $CD34^+/HLA\text{-}DR^-$ cells were cultured in transwells above AFT024 and M2-10B4 feeders for 5 weeks and the absolute frequency of LTC-IC maintained after 5 weeks culture was assessed by LDA on M2-10B4 feeders. In M2-10B4 non-contact cultures, 36.6 ± 4.3% LTC-IC were maintained for 5 weeks. However, only 15.0 ± 2.6% of LTC-IC were maintained in AFT024 non-contact cultures (Figure 1) ($P = 0.02$, $n = 6$). This led to the following hypotheses: (1) Decreased support of primitive progenitors in AFT024 non-contact cultures is due to inhibitory factors released by AFT024 cells; or (2) factors crucial for maintenance of primitive human progenitors, such as proteoglycans or cytokines, are not secreted by AFT024 cells.

To investigate if inhibitory factor(s) secreted by AFT024 cells are responsible for the poor maintenance of LTC-IC in AFT024 non-contact cultures, we added AFT024 conditioned medium to M2-10B4 non-contact cultures for the 5 week culture duration. LTC-IC-maintenance in M2-10B4 non-contact cultures supplemented with AFT024 conditioned medium was not significantly lower than that seen in M2-10B4 non-contact cultures maintained in LTBMC-medium (50.7 ± 9.4%, $n = 2$ *vs* 36.6 ± 4.33%, $n = 6$ NS). Thus, poor LTC-IC-maintenance seen in AFT024 non-contact cultures is not caused by secretion of inhibitory factors (Figure 1).

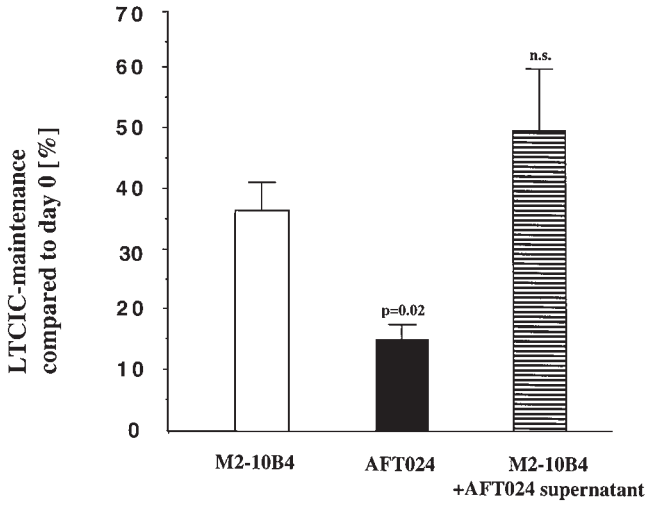

Figure 1 Poor support of LTC-IC in AFT024 non-contact cultures is not due to presence of inhibitory or differentiating factors in the supernatant. 10 000 $CD34^+/HLA\text{-}DR^-$ cells were cultured for 5 weeks in transwells above AFT024 ($n = 6$) or M2-10B4 ($n = 6$) feeders. For some M2-10B4 cultures medium, exchanges were done using LTBMC-medium conditioned for 48 h on confluent AFT024 feeders ($n = 2$). Maintenance of LTC-IC was determined by comparing the absolute number of LTC-IC on day 0 with the absolute number of LTC-IC at week 5. Statistical analysis: results are shown as mean ± s.e.m. Comparison made between M2-10B4 cultures and AFT024 cultures ($P = 0.02$; paired *t*-test), or M2-10B4 cultures and M2-10B4 cultures + AFT024 conditioned medium cultures (n.s.; unpaired *t*-test).

We have previously shown that 6-O-sulfated HS-GAGs secreted by M2-10B4 are important for LTC-IC maintenance in M2-10B4 non-contact cultures.[16,17] Poor LTC-IC maintenance in FHS-173-We non-contact cultures can at least in part be explained by absence of such HS-GAGs.[3,16,17] We therefore characterized the type of GAGs produced by AFT024 feeders and compared them with M2-10B4-HS-GAGs (Table 1). The average size of HS-GAGs in the conditioned medium of AFT024 cells was 34 kDa, not different from HS-GAGs secreted in M2-10B4 conditioned medium (45 kDa). Analysis of the sulfation pattern of HS-GAGs in AFT024 and Ms-10B4 conditioned media showed that a comparable proportion of ^3H was present in the oligosaccharides (3–8 monosaccharides) obtained by nitrous acid degradation of HS-GAGs from the conditioned medium of both cell lines. This indicates that a large but comparable proportion of the polysaccharide backbone of HS-GAGs from both cell lines is comprised of such regions. The proportion of ^{35}S present in these oligosaccharides was also comparable for AFT024 and M2-10B4 HS-GAGs indicating that the HS-GAGs from both cell lines have an equivalent extent of O-sulfation, largely as 6-O-sulfate. Thus, the size and extent of O-sulfation of HS-GAGs in AFT024 is similar to the LTC-IC supportive HS-GAGs present in M2-10B4 and differences in HS-GAGs secreted in AFT024 conditioned medium do not account for the lack of LTC-IC supportive capacity of AFT024-conditioned medium.

We next investigated if poor support of LTC-IC in AFT024 non-contact cultures is due to lack of certain cytokines. We

Table 1 Size and sulfation of heparan sulfate (HS) from AFT024 and M2-10B4 supernatants

	AFT024	*M2-10B4*
Average size of HS (kDa)	34	45
Analysis of O-sulfated oligosaccharides of HS		
^{3}H (proportion of total polysaccharide backbone[a]	49 ± 1%	40 ± 0%
^{35}S (proportion of total sulfate)[a]	24 ± 1%	27 ± 0%

Analysis of HS obtained from one representative purification procedure are shown. Similar figures were obtained for size and sulfation in additional experiments. The average size of HS is that of the major peak in each sample.
[a]Proteoglycans in these samples eluted as two to three separate peaks on anion exchange HPLC purification. HS was purified separately from each peak, digested using nitrous acid and analyzed for O-sulfation pattern by gel filtration chromatography. The pattern of O-sulfation was similar in HS in the various HPLC peaks from each sample. Data are shown as the mean ± s.e.m. of the analysis of the individual peaks.

first analyzed, by ELISA, levels of the murine cytokines IL-1β, IL-3, IL-6, IL-10, IL-12p40, IL-12p70, GN-CSF, MIP-1α, MIP-1β, MIP-2, TNFα, TPO and SCF in M2-10B4 and AFT024 non-contact cultures. All cytokines, except SCF, either in AFT024 or M2-10B4 conditioned medium were present at levels below the sensitivity threshold for each individual test. The levels for murine SCF were not significantly different in AFT024-conditioned media (150.5 ± 9.5 pg/ml) and M2-10B4-conditioned media (144 ± 13 pg/ml).

We have previously shown that a combination of six human cytokines at concentrations, found in marrow stromal cultures[3] combined with M2-10B4 specific HS-GAGs support 40% of LTC-IC for 5 weeks.[16,17] We therefore added these six cytokines to AFT024 non-contact cultures. After 5 weeks, 71.9 ± 8.8% ($n = 6$) of LTC-IC were maintained in cytokine supplemented AFT024 non-contact cultures (Figure 2), which was significantly better than without cytokines (15.0 ± 2.59%, $P < 0.001$). To evaluate if a single cytokine was responsible for the increase in LTC-IC maintenance, we cultured $CD34^{+}/HLA\text{-}DR^{-}$ cells in AFT024 non-contact cultures supplemented with five of the six cytokines (Figure 2). Although, LTC-IC maintenance was slightly lower following the removal of each of the six cytokines, the decrease was only significant when G-CSF (65.1 ± 11.0%), $P = 0.03$), MIP-1α (59.9 ± 5.8%, $P = 0.002$) or SCF (62.7 ± 8.0%, $P = 0.02$) were removed.

To determine if the cytokines act on the progenitors directly we cultured $CD34^{+}/HLA\text{-}DR^{-}$ cells in stroma-free wells and supplemented the cultures with AFT024 conditioned medium to which the combination of the six cytokines was added (Figure 3). Conditioned medium from AFT024 without the six human cytokines did not support LTC-IC (<1% maintenance). When the six cytokines were added to AFT024-conditioned medium, 30.3 ± 7.45% of LTC-IC were maintained. We also cultured $CD34^{+}/HLA\text{-}DR^{-}$ cells in BSA-coated wells in cytokine-conditioned AFT024 medium. After 5 weeks of culture 50.8 ± 4.8% ($n = 3$) of LTC-IC were maintained. Thus, six human cytokines act in part direct on LTC-IC and in part indirect through the AFT024 feeder. Of interest, LTC-IC maintenance was significantly better in AFT024 non-contact cultures supplemented with the six cytokines (77.2 ± 3.4%) than in cytokine-conditioned AFT024 medium cultures (50.8 ± 4.8%, $P = 0.04$).

Discussion

We and others have recently shown that human LTC-IC, primitive NK progenitors, and multilineage progenitors can be maintained and expanded when cultured in contact with AFT024 feeders.[12,13,21] We now show that LTC-IC are poorly supported when cultured in non-contact conditions. This is consistent with a recent report by Thiemann *et al*[14] showing that $CD34^{+}/CD38^{-}$ cord blood progenitors cultured in transwells above AFT024 feeders are not maintained. We investigated the mechanism underlying this observation which led to a number of conclusions.

First, the addition of AFT024-conditioned medium did not inhibit LTC-IC maintenance in M2-10B4 non-contact cultures. This shows that the poor LTC-IC maintenance in AFT024 non-contact cultures cannot be explained by the secretion of factors that inhibit LTC-IC or induce their differention in AFT024-conditioned medium.

Second, we have previously shown that although LTC-IC are maintained to the same extent when cultured in contact with the cell lines M2-10B4, FHS-173-We, NIH3T3 or primary human marrow stroma,[3] LTC-IC are not well maintained in FHS-173-We and NIH3T3 non-contact cultures.[3] Differences in the ability of FHS-173-We and M2-10B4 or primary marrow stroma-conditioned medium to support LTC-IC can at least in part be attributed to differences in proteoglycans secreted by the different feeders. 6-O-sulfated HS-GAGs which are required for LTC-IC maintenance are secreted by M2-10B4 but not FHS-173-We cells.[16,17] We therefore characterized the structure and sulfation pattern of HS-GAGs present in AFT024-conditioned medium cultures. AFT024 feeders secrete HS-GAGs with similar structure and sulfation pattern to HS-GAGs secreted by MS-10B4 feeders known to be important for LTC-IC maintenance. This indicates that factor(s) other than HS-GAGs must be responsible for the poor LTC-IC support in AFT024 non-contact cultures.

Third, we have shown that pg concentrations of at least six different cytokines found in bone marrow stromal supernatants are required in combination with 6-O-sulfated HS-GAGs for LTC-IC maintenance.[16,17] We performed ELISAs for murine cytokines in AFT024- and M2-10B4-conditioned medium and could not detect differences in measurable levels of murine cytokines in AFT024 and M2-10B4 conditioned media. Thiemann *et al*[14] measured levels of Flt3 ligand in AFT024 and primary marrow stromal supernatants. Levels of Flt3 ligand were not significantly different in the two supernatants. Further, Wineman *et al*[22] and Moore *et al*[23] showed that mRNA levels of more than 15 known cytokines were not different in AFT024 cells from that seen in non-supportive AFT clones. It is therefore not likely that differences in known cytokines can explain the poor LTC-IC maintenance seen in AFT024 non-contact cultures.

To further address the possibility that a growth factor with activity on human progenitors was lacking in AFT024-conditioned medium we added human GM-CSF, G-CSF, SCF, LIF, MIP-1α and IL-6 in concentrations found in marrow stromal cultures.[3,16,17] Somewhat surprisingly, we found that more than 70% of LTC-IC could be maintained in AFT024 non-contact cultures supplemented with the combination of these six cytokines. We attempted to identify the cytokine(s) present in the combination which is responsible for the supportive effect.

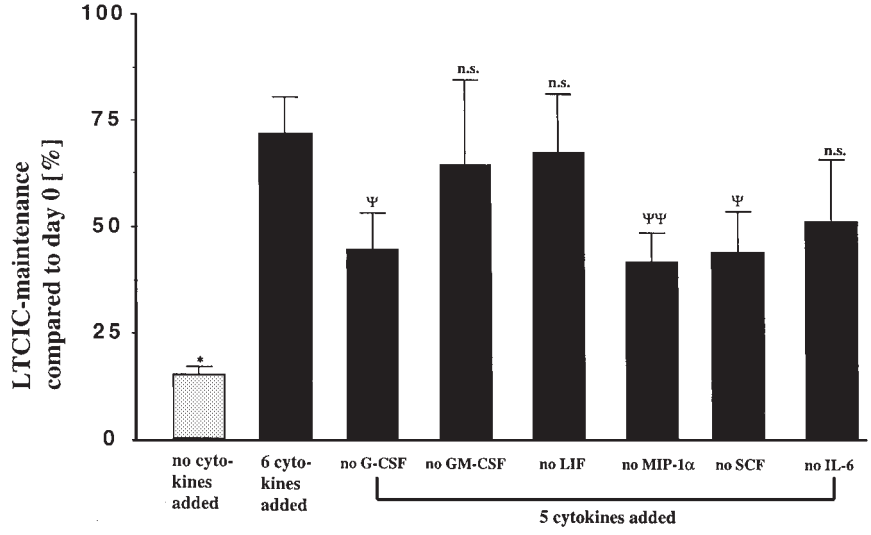

Figure 2 Addition of six human cytokines in concentrations found in primary marrow stroma increases LTC-IC maintenance in AFT024 non-contact cultures significantly. 10 000 $CD34^+/HLA\text{-}DR^-$ cells were cultured for 5 weeks in transwells above AFT024 feeders with LTBMC-medium without ($n = 6$), or with a combination of six human cytokines ($n = 6$), or with a combination of five human cytokines, omitting one of the six cytokines per experimental point as indicated ($n = 3$–5). Maintenance of LTC-IC was determined by comparing the absolute number of LTC-IC on day 0 with the absolute number of LTC-IC at week 5. $^*P < 0.001$, comparison between AFT024 non-contact cultures without and with the six human cytokines (unpaired *t*-test). $^{\Psi}P < 0.05$, $^{\Psi\Psi}P < 0.01$, comparison between AFT024 non-contact cultures with six or with five cytokines (paired *t*-test).

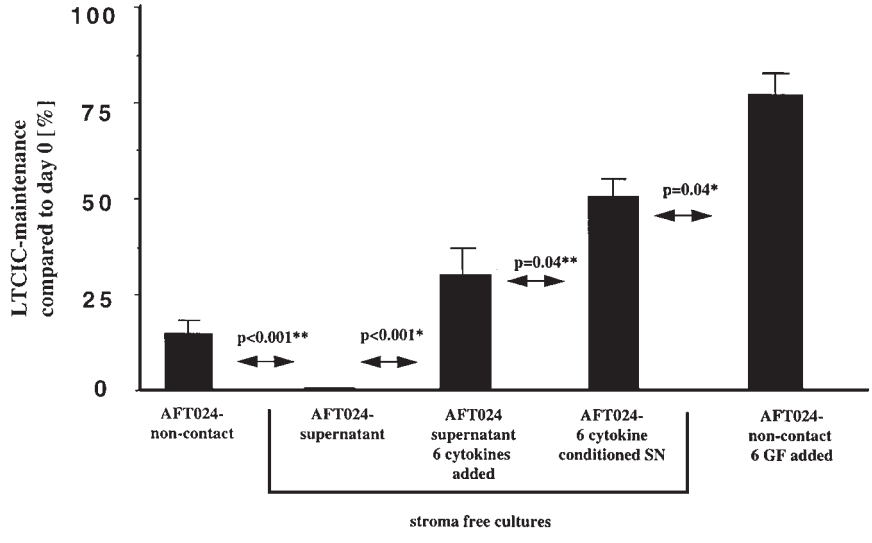

Figure 3 Improved LTC-IC maintenance caused by the addition of six human cytokines is in part related to direct effects on LTC-IC and in part caused by indirect effects through the AFT024 feeder. 10 000 $CD34^+/HLA\text{-}DR^-$ cells were cultured in AFT024 non-contact cultures with ($n = 3$) and without ($n = 6$) the addition of six human cytokines. Alternatively, 10 000 $CD34^+/HLA\text{-}DR^-$ cells were cultured in stroma-free wells and supplemented with AFT024-conditioned medium ($n = 4$), AFT024 conditioned medium supplemented with six cytokines ($n = 3$), or cytokine-conditioned AFT024 medium ($n = 3$). Maintenance of LTC-IC was determined by comparing the absolute number of LTC-IC on day 0 with the absolute number of LTC-IC at week 5 (*paired *t*-test, **unpaired *t*-test).

Removal of either SCF, G-CSF or MIP-1α from the combination decreased LTC-IC maintenance significantly. However, the effect of removing one cytokine was only partial and culture of $CD34^+/HLA\text{-}DR^-$ cells in AFT024 non-contact cultures with five of the six cytokines continued to be significantly better than without cytokines. Therefore the question of whether a single cytokine is responsible for the improved LTC-IC maintenance remains unanswered.

LTC-IC maintenance in stroma-free cultures supplemented with AFT024 conditioned medium and the six cytokines was significantly better than without the cytokines and was enhanced further in cultures maintained with cytokine-conditioned AFT024 medium. Breems *et al*[10] have shown that supernatants from the cell lines L88/5, L87/4 and F8MD-1 significantly increase week 6–8 CAFC expansion. We now demonstrate that the LTC-IC supportive effect of AFT024-conditioned medium enhanced significantly following addition of cytokines to the medium prior to conditioning on the feeder. This demonstrates that the improved LTC-IC support caused by cytokines is only in part due to direct effects on the progenitors and in part to indirect effects through the stroma. ELISAs of conditioned medium from AFT024 non-contact cul-

tures supplemented with either all six human cytokines or five of the six cytokines did not reveal increased secretion of any of the measured murine cytokines (data not shown). The nature of the factor(s) made by AFT024 responsible for the indirect effect of the human cytokines on LTC-IC maintenance remains therefore unknown.

Finally, LTC-IC maintenance in AFT024 non-contact cultures supplemented with the six human cytokines was significantly better than in cytokine-conditioned AFT024 medium cultures. Likewise, significantly more LTC-IC were maintained in AFT024 non-contact cultures without cytokines compared to AFT024-conditioned medium cultures. This illustrates the complex nature of optimal LTC-IC maintenance. The requirement of progenitor stroma cross talk shown here is consistent with studies recently reported by our group and others demonstrating that soluble factor(s) released by $CD34^+$ cells influence the expression of certain cytokines which may be important for progenitor growth.[24–26] We were unable to demonstrate differences in cytokine levels in supernatants of AFT024 feeders cultured with or without human $CD34^+$ cells. Therefore, the nature of the factor(s) induced by the progenitor-stroma coculture also still needs to be characterized.

In conclusion we show that addition of cytokines or cells, or even more cytokines and cells to AFT024 cultures enhances the ability of AFT024-conditioned medium to support LTC-IC. This will now be exploited to identify novel growth factors responsible for LTC-IC maintenance or expansion.

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The Myeloid-Lymphoid Initiating Cell (ML-IC) Assay Assesses the Fate of Multipotent Human Progenitors In Vitro

By M. Punzel, S.D. Wissink, J.S. Miller, K.A. Moore, I.R. Lemischka, and C.M. Verfaillie

Hematopoietic stem cells (HSC) are cells with self-renewing multilineage differentiation potential. Although engraftment in xenogeneic recipients can be used to measure human HSC, these assays do not allow assessment of individual progenitors. We developed an in vitro assay that allows the identification of a single human bone marrow progenitor closely related to HSC, which we termed "Myeloid-Lymphoid Initiating Cell," or ML-IC, because it is capable of generating multiple secondary progenitors that can reinitiate long-term myeloid and lymphoid hematopoiesis in vitro. The assay is done in contact with murine AFT024 fetal liver stromal cells and with Flt3-Ligand, stem cell factor, and interleukin-7. In this assay, 0.2% to 1.7% of Lin $^-$/34$^+$/DR^{dim} cells could generate 1 to 3 long-term culture initiating cells (LTC-IC) as well as 1 to 4 NK-IC after 4 to 6 weeks. In addition, this assay measures contribution of net-progenitor conservation and net-progenitor proliferation over time, providing insight in the fate of individual LTC-IC and NK-IC. This assay will prove useful to enumerate the number of very primitive human progenitors with multilineage differentiation potential, as well as to evaluate future ex vivo culture conditions.

HEMATOPOIETIC STEM cells (HSC) are defined as cells capable of self renewal as well as multilineage differentiation. In mice, the phenotype and function of HSC have been characterized using competitive in vivo repopulation assays.[1-4] Because such repopulation assays cannot be performed in humans, surrogate in vivo or in vitro assays are used to evaluate human HSC. Transplantation of human progenitors in xenogeneic transplant recipients, such as severe combined immunodeficient (SCID) mice,[5-7] beige-nude-SCID (BNX)-mice,[8,9] nonobese diabetic (NOD)-SCID-mice,[10-13] or fetal sheep,[14,15] allows detection of engrafting human cells. In vivo production of myeloid, natural killer (NK), T-lymphoid, and B-lymphoid blood elements is seen for several months to years after transplantation. Through transplantation of limiting numbers of CD34$^+$ subpopulations, these xenotransplant models provide a quantitative assay for engrafting cells.

A number of in vitro assays have been described that assess primitive human progenitors. These include long-term culture initiating cell (LTC-IC) assays,[16] cobblestone area forming cell (CAFC) assays,[17,18] and extended (E)-LTC-IC assays.[19] These assays enumerate primitive progenitors that can eventually generate myeloid cells, but not cells with multilineage differentiation or self-renewal potential. Several groups have developed cultures that allow differentiation of single human CD34$^+$Lin$^-$ cells into cells with myeloid, NK, B-lymphoid, dendritic, and/or T-lymphoid phenotype showing that a single cell can differentiate in vitro into multiple lineages.[20-22] Because of its multilineage differentiation capacities, this cell is thought to be more immature than the LTC-IC.

In the work described here, we wanted to develop an assay that would allow us to enumerate even more primitive cells that, besides having multilineage differentiation potential, also can generate secondary primitive progenitors that again have multilineage differentiation potential. A number of observations prompted us to use a stroma-based culture system to accomplish this. Long-term ex vivo maintenance of murine HSC also requires stroma.[23] It has also been shown that noncommitted human progenitors require stromal cell interactions that cannot be replaced by soluble factors to commit to the B-lymphoid lineage.[24-27] Our group has recently shown that human CD34$^+$/HLA-DR$^-$/Lin$^-$ bone marrow progenitors require stromal contact to differentiate into functional NK cells.[28]

We used the murine fetal liver cell line, AFT024, known to support murine repopulating HSC for up to 7 weeks ex vivo,[23] as well as human primitive myeloid and lymphoid progenitors.[29,30] We show that a culture system based on this AFT024 feeder, supplemented with the early acting cytokines Flt3-Ligand (Flt3-L), stem cell factor (SCF), and interleukin-7 (IL-7), can enumerate very primitive human progenitors capable of generating multiple secondary progenitors that have the ability of reinitiating long-term multilineage hematopoiesis. We termed these cells Myeloid-Lymphoid Initiating Cells or ML-IC. This single cell assay also provides insight in the mechanisms underlying ex vivo expansion of primitive myeloid and lymphoid progenitors.

MATERIALS AND METHODS

Cell Source and Preparation

Bone marrow was aspirated from the posterior iliac crest from healthy volunteer donors after obtaining informed consent using guidelines approved by the Committee on the Use of Human Subjects at the University of Minnesota (Minneapolis, MN). Mononuclear cells (MNC) were obtained by Ficoll-Hypaque (Sigma-Diagnostics, St Louis, MO) centrifugation. CD34$^+$ cells were selected with a biotinylated monoclonal anti-CD34 antibody (clone, 12.8 CePrate system; CellPro Inc, Bothell, WA) on an immunoaffinity column (CellPro).

From the Department of Medicine, Division of Hematology and Stem Cell Biology Program, University of Minnesota, Minneapolis, MN; and the Department of Molecular Biology, Princeton University, Princeton, NJ.

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Address reprint requests to C.M. Verfaillie, MD, Professor of Medicine, Division of Hematology, Oncology and Transplantation, Director, Stem Cell Biology Program, Box 806 UMHC, 420 Delaware St SE, Minneapolis, MN 55455; e-mail: verfa001@maroon.tc.umn.edu.

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Fluorescence-Activated Cell Sorting (FACS)

$CD34^+$ enriched cells were incubated with anti-CD34-Biotin (CellPro), Streptavidin-SA670 (GIBCO-BRL, Grand Island, NY), anti-HLA-DR-phycoerythrin (PE) (Becton Dickinson, Mountain View, CA), and a lineage cocktail of fluorescein isothiocyanate (FITC)–conjugated antibodies against CD2, CD3, CD4, CD5, CD7, CD8, CD10, CD14, CD15, CD16, CD19 (Becton-Dickinson). Individual $Lin^-/34^+/DR^{dim}$ cells were sorted into 96-well plates (Costar, Cambridge, MA) containing irradiated AFT024 feeders using the automatic cell deposition unit (ACDU) on a FACS Star-Plus flow cytometry system equipped with a Consort 32 computer (Becton Dickinson). To ensure that only a single cell was deposited, the ACDU was set up in a low event "through-put" (200 cells/s). An oscilloscope was used to adjust phasing of the single droplet sort pulse to the drop drive frequency. Accuracy of the single-cell deposition was determined by sorting single cells in 96-well plates without stroma and scoring wells visually for the number of cells deposited: 83% of the wells contained a single cell, 17% of the wells did not contain a cell, and no well contained more than one cell.

Stromal Feeder

The murine fetal liver cell line, AFT024, was maintained at 33°C in Dulbecco's modified Eagle's medium (DMEM; GIBCO-BRL) supplemented with 20% fetal calf serum (FCS; Hyclone Laboratories, Logan, UT), 50 μmol/L 2-Mercaptoethanol (2-ME; Bio-Rad, Hercules, CA). AFT024 cells were subcultured in 24- or 96-well plates (Costar), precoated with 0.1% gelatin (Specialty Media, Lavalette, NJ), and grown to confluence. Confluent plates were irradiated at 2,000 rad and were maintained at 37°C.

Culture Media

Flt3-L/SCF/IL-7 expansion medium. RPMI 1640 (GIBCO-BRL), 20% FCS, 25 μmol/L 2-ME, 1,000 U/mL penicillin, 100 U/mL streptomycin (GIBCO-BRL), Flt3-L (10 ng/mL; Amgen, Thousand Oaks, CA), SCF (10 ng/mL; Immunex, Seattle, WA), and IL-7 (20 ng/mL; R&D Systems, Minneapolis, MN).

IL-3/MIP-1α expansion medium. Iscove's modified Dulbecco's medium (IMDM), 12.5% FCS, 12.5% horse serum (Stem Cell Technologies, Vancouver, Canada), 2 mmol/L L-Glutamine (GIBCO-BRL), 1,000 U/mL penicillin, 100 U/mL streptomycin, 10^{-6} mol/L hydrocortisone, IL-3 (5 ng/mL), and macrophage inflammatory protein (MIP)-1α (100 ng/mL; R&D Systems).

Lymphoid differentiation medium. DMEM and Ham's F12-medium (GIBCO-BRL) in a 2:1 (vol/vol) mix containing 20% heat inactivated human AB serum (North American Biologicals, Miami, FL), ascorbic acid (20 mg/mL; GIBCO-BRL), selenium selenite (50 μmol; GIBCO-BRL), 2-ME (25 μmol), and ethanolamine (50 μmol; GIBCO-BRL). Cytokines added on day 0: IL-2 (1,000 U/mL; Amgen), IL-3 (5 ng/mL), IL-7 (20 ng/mL), SCF (20 ng/mL), and Flt3-L (10 ng/mL). At weekly intervals, half media change was done using 10% instead of 20% human AB serum. Cytokines added at week 1 and later: IL-2 only (1,000 U/mL).

Single Cell Cultures

To determine the LTC-IC frequency on day 0, cells were sorted individually in 96-well plates ("day 0 plates"), maintained either in AFT024/Flt3-L/SCF/IL-7 or AFT024/IL-3/MIP-1α conditions for 5 weeks, and then overlaid with clonogenic methylcellulose medium (Methylcellulose [Fisher, Chicago, IL] in a final concentration of 1.12% containing IMDM, supplemented with 30% FCS, 3 IU/mL erythropoietin [Amgen], and supernatant of the bladder carcinoma cell line 5637 [7.5%]) and scored for secondary colony-forming cell (CFC) after an additional 2 weeks. To determine the NK-culture-initiating cell (IC) frequency on day 0, single cell cultures were maintained with lymphoid differentiation medium for 5 to 6 weeks. Wells were scored visually for presence of mature progeny. To demonstrate presence of NK cells, wells were harvested and cells were stained with anti-CD56-PE and anti-CD3-FITC (Becton Dickinson) and analyzed by FACS to determine the presence of $CD56^+$ NK cells. We also tested secondary $CD56^+$ NK cells for their capability to kill specifically K562 targets as previously described.[31]

To assess ML-IC, cells were sorted individually in 96-well plates ("expansion cultures") and maintained in either AFT024/Flt3-L/SCF/Il-7 or AFT024/IL-3/MIP-1α expansion conditions for 4 weeks. The content of a single well was then harvested with trypsin and divided equally over 8 secondary 96-well plates containing irradiated AFT024 feeders in such a manner that one eighth of each single cell progeny was deposited in the identical location in the 8 secondary plates. Four of the 8 secondary plates were maintained in either AFT024/Flt3-L/SCF/IL-7 or AFT024/IL-3/MIP-1α expansion conditions for an additional 5 weeks and assessed for LTC-IC as described above. The other 4 secondary plates were maintained for 6 to 7 weeks with lymphoid differentiation medium and assessed for the presence of NK cells by FACS. LTC-IC and NK-IC expansion was determined as described in Table 1.

In other experiments, individually sorted $Lin^-/34^+/DR^{dim}$ cells were cultured in Flt3-L/SCF/IL-7 expansion medium on AFT024 feeders. After 3 weeks, the primary plates were harvested by trypsinization, and the contents of each well were divided equally into 4 secondary 96-well plates in such a manner that one fourth of the progeny of each single cell was deposited in the identical location in the 4 secondary plates. Secondary cultures were maintained for an additional 3 weeks in Flt3-L/SCF/IL-7 expansion medium. After 6 weeks, secondary plates were harvested by trypsinization, and the contents of each well were divided equally into 4 tertiary 96-well plates in such a manner that one fourth of the content of each secondary well was deposited in the identical location of the 4 tertiary plates. Two of the tertiary plates (8 for each primary plate) were assessed for LTC-IC, and two of the tertiary plates (8 for each primary plate) were assessed for NK-IC as described above.

Table 1. LTC-IC Expansion in AFT024/Flt3-L/SCF/IL-7 Cultures Is Due to LTC-IC Net-Conservation and Net-Proliferation

		LTC-IC at Week 4						
			No Proliferation		Proliferation			
Exp.	Day 0 LTC-IC (%)	Conserved LTC-IC	1 LTC-IC	2 LTC-IC	3 LTC-IC	4 LTC-IC	Total LTC-IC	Fold Expansion
1	6.9%	11/132* (8.3%)	4/11†	4/11	2/11	1/11	22/132‡ (16.6%)	2.4
2	8.5%	13/132* (9.8%)	8/13†	4/13	0/13	1/13	20/132‡ (15.2%)	1.8
3	6.8%	11/88* (12.5%)	5/11†	5/11	1/11	0/11	18/88‡ (20.4%)	3.0
4	4.2%	6/88* (6.8%)	4/6†	1/6	0/6	1/6	10/88‡ (11.4%)	2.7

*Number of single-sorted $Lin^-/34^+/DR^{dim}$ cells that generated at least 1 secondary LTC-IC per initially plated cell.

†Number of LTC-IC (per conserved LTC-IC) that did not proliferate (only 1 secondary LTC-IC).

‡Total number of secondary LTC-IC per single sorted $Lin^-/34^+/DR^{dim}$ cell.

Definitions

Overall progenitor expansion is equal to recovery of >100% LTC-IC/NK-IC in all week 4 or 6 progeny plates compared with the day 0 plates. Progenitor proliferation is equal to a single cell that gives rise to two or more LTC-IC/NK-IC, ie, can reinitiate hematopoiesis in at least 2 secondary plates. Progenitor conservation is equal to a single cell that persists over time with or without proliferation, ie, can reinitiate hematopoiesis in at least one secondary LTC-IC/NK-IC culture. Progenitor recruitment is equal to a single cell that reads out as an LTC-IC/NK-IC after 4 to 6 weeks, but not on day 0.

Statistics

Results of experimental points from different experiments were reported as the mean ± standard error of the mean (SEM). Significance levels were determined by either paired or nonpaired two-sided Student's *t*-test analysis as indicated.

RESULTS

Measurement of Multilineage Initiating Cells

To determine if a single cell can give rise to secondary progenitors with both LTC-IC and NK-IC characteristics, we cultured single Lin$^-$/34$^+$/DR^{dim} cells from 4 individual donors (176 to 264 cells per donor) in Flt3-L/SCF/IL-7 expansion medium on AFT024 feeders. After 4 weeks, one eighth of the contents of each individual well was replated in the identical location of 8 secondary AFT024-containing 96-well plates. Four of the 8 secondary plates were maintained for 5 weeks in Flt3-L/SCF/IL-7 expansion medium and then overlaid with clonogenic methylcellulose medium to assess presence of LTC-IC in single-cell progeny. To assess NK-IC, the other 4 plates were maintained under lymphoid differentiation condi-

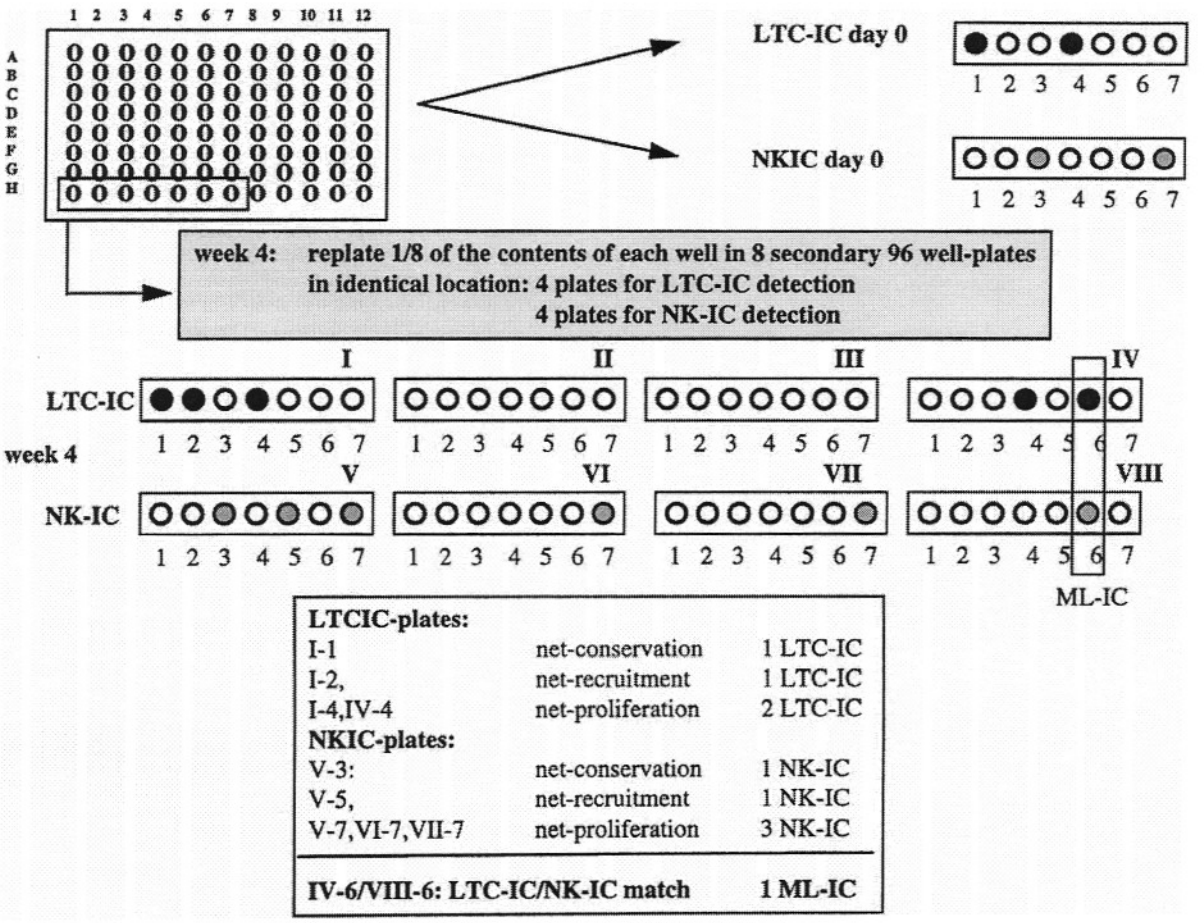

Fig 1. Single cell cultures. Lin$^-$/34$^+$/DR^{dim} cells were sorted into 88 wells of 96-well plates with AFT024 stromal feeders using the FACS Star Plus ACDU system. Cells were cultured as indicated with weekly medium exchange. After 4 weeks, the content of each well was collected by trypsinization and divided equally over 8 secondary 96-well plates with pre-established AFT024 feeders in such a manner that one eighth of the content was deposited in the identical location in the 8 secondary plates, 4 for LTC-IC detection and 4 for NK-IC detection. To detect LTC-IC/NK-IC at day 0, single cells were cultured in 96-well plates containing AFT024 feeders for 5 weeks, and plates were overlaid with clonogenic methylcellulose. To detect NK-IC at day 0, single cells were cultured in separate 96-well plates containing AFT024 feeders as described in Materials and Methods. An ML-IC is defined as a single Lin$^-$/34$^+$/DR^{dim} cell with multilineage generative capacity, ie, this cell can generate at least one LTC-IC and one NK-IC.

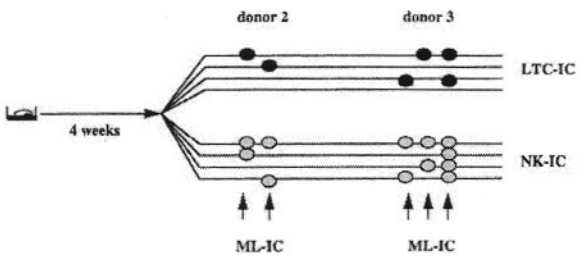

Fig 2. 0.8% to 1.7% of Lin$^-$/34$^+$/DR^{dim} cells are myeloid-lymphoid initiating cells. Progeny from single Lin$^-$/34$^+$/DR^{dim} cells cultured on AFT024 in expansion medium with Flt3-L, SCF, and IL-7 were replated after 4 weeks in 8 secondary plates. Four secondary plates were maintained in expansion medium with Flt3-L, SCF, and IL-7 to enumerate LTC-IC. The remaining 4 secondary plates were maintained in lymphoid differentiation medium to detect NK-IC. Lin$^-$/34$^+$/DR^{dim} cells that generated ≥1 LTC-IC (black circles) and ≥1 NK-IC (gray circles) were identified as ML-IC.

tions for 6 to 7 weeks (Fig 1). Detection of ≥1 LTC-IC and ≥1 NK-IC in the identical location in secondary plates was required to consider the initially plated Lin$^-$/34$^+$/DR^{dim} cell an ML-IC.

In 2 out of 4 donors, we found that 0.8% and 1.7% of Lin$^-$/34$^+$/DR^{dim} cells could generate both secondary LTC-IC and NK-IC after the initial 4-week expansion culture, or were ML-IC. Each initially sorted ML-IC gave rise to 1 or 2 LTC-IC, as well as 2 to 4 NK-IC. In the other 2 donors, such cells were not detected (Fig 2). In additional experiments, we extended the initial culture in Flt3-L/SCF/IL-7 expansion medium to 6 weeks (data not shown). We detected ML-IC in 3 of 4 donors. The frequency of ML-IC ranged between 0.2% and 0.7%, and each ML-IC generated 1 to 3 LTC-IC and 1 NK-IC.

Net-Conservation and Net-Proliferation Contribute to LTC-IC and NK-IC Expansion in Flt3-L/SCF/IL-7 Expansion Medium Cultures

We hypothesized that this single cell assay with LTC-IC and NK-IC readout assays would also provide information on the fate of a single LTC-IC or NK-IC progenitor over time; specifically, if an LTC-IC or NK-IC persists over time or if there is net conservation, and if a single cell can give rise to two or more LTC-IC or NK-IC (net-proliferation) during the primary culture step.

Single Lin$^-$/34$^+$/DR^{dim} cells were cultured in Flt3-L/SCF/IL-7 expansion medium for 4 weeks, and progeny were evaluated for LTC-IC and NK-IC, as described above. The absolute number of Lin$^-$/34$^+$/DR^{dim} cells capable of initiating long-term cultures on day 0 was 6.6% ± 0.9%. Of the initially plated Lin$^-$/34$^+$/DR^{dim} cells, 9.3% ± 1.2% were capable of initiating and sustaining hematopoiesis in one or more secondary stromal cultures. Thus, culture for 4 weeks in Flt3-L/SCF/IL-7 expansion medium on AFT024 feeders supported net conservation of LTC-IC. Cultured progeny of 47.5% ± 7% of single Lin$^-$/34$^+$/DR^{dim} LTC-IC were able to initiate hematopoiesis in 2, 3, or 4 secondary long-term cultures and had therefore proliferated. The overall LTC-IC frequency after 4 weeks, or all LTC-IC present in the 4 secondary cultures, was 15.9% ± 1.8%, or 2.5 ± 0.2–fold higher than that measured in day 0 Lin$^-$/34$^+$/DR^{dim} cells (Table 1). In three additional experiments, all 8 secondary plates were evaluated for the presence of secondary LTC-IC. The maximal number of secondary LTC-IC generated from one individual cell was 6.

To examine the fate of single NK-IC, progeny of the primary Flt3-L/SCF/IL-7 expansion medium cultures were replated in contact with AFT024 feeders with lymphoid differentiation medium for 6 to 7 weeks. The NK-IC frequency in freshly sorted Lin$^-$/34$^+$/DR^{dim} cells measured by culturing for 6 to 7 weeks in lymphoid differentiation medium was 0.12% ± 0.02%. In 3 of 4 experiments, progeny of 3.9% ± 1.7% of the initial Lin$^-$/34$^+$/DR^{dim} cells cultured for 4 weeks in expansion medium were capable of generating functional NK cells in one or more secondary lymphoid differentiation cultures indicating net conservation of NK-IC (Table 2). 46% ± 26% of single-sorted Lin$^-$/34$^+$/DR^{dim} cells that generated NK-IC after 4 weeks could initiate 2 to 4 secondary NK long-term cultures, indicating proliferation. Thus, a total of 33 NK-IC (7.5% ± 3.7%) was present in all 4 secondary cultures initiated with progeny of 440 Lin$^-$/34$^+$/DR^{dim} cells, which is 37.3 ± 19–fold higher than the day 0 frequency of NK-IC.

Lack of Progenitor Proliferation Is Responsible for Lack of LTC-IC and NK-IC Expansion in IL-3/MIP-1α Expansion Medium Cultures

To confirm that this assay will be helpful in assessing the contribution of net proliferation and net conservation of progenitors to overall progenitor expansion, we cultured single-sorted Lin$^-$/34$^+$/DR^{dim} cells from 5 donors in contact with AFT024 feeders under conditions known to maintain LTC-IC, namely IL-3/MIP-1α expansion medium (unpublished observations). We hypothesized that our assay should show that lack of expansion is caused by either poor conservation or poor

Table 2. NK-IC Expansion in AFT024/Flt3-L/SCF/IL-7 Cultures Is Due to NK-IC Net-Conservation and Net-Proliferation

		NK-IC at Week 4						
			No Proliferation	Proliferation				
Exp.	Day 0 NK-IC (%)	Conserved NK-IC	1 NK-IC	2 NK-IC	3 NK-IC	4 NK-IC	Total NK-IC	Fold Expansion
1	<0.2%	3/132* (2.3%)	3/3†	0/3	0/3	0/3	3/132‡ (2.2%)	>11
2	<0.2%	9/132* (6.8%)	2/9†	6/9	1/9	0/9	17/132‡ (12.9%)	>64
3	<0.2%	6/88* (6.8%)	1/6†	4/6	0/6	1/6	13/88‡ (14.8%)	>74
4	<0.2%	0/88* (<1.1%)	0	0	0	0	0/88‡ (<1.1%)	NE

Abbreviation: NE, not evaluated.

*Number of single-sorted Lin$^-$/34$^+$/DR^{dim} cells that generated at least 1 secondary NK-IC per initially plated cell.

†Number of NK-IC (per conserved/recruited NK-IC) that did not proliferate (only 1 secondary NK-IC).

‡Total number of secondary NK-IC per single sorted Lin$^-$/34$^+$/DR^{dim} cell.

proliferation of LTC-IC and NK-IC. After culture of single $Lin^-/34^+/DR^{dim}$ cells for 4 weeks in IL-3/MIP-1α expansion medium, the contents of each well of the primary cultures were replated in 8 secondary AFT024-containing 96-well plates to assess presence of LTC-IC (4 plates) and NK-IC (4 plates). On day 0, 7.7% ± 1.5% of $Lin^-/34^+/DR^{dim}$ cells had LTC-IC characteristics. After 4 weeks in IL-3/MIP-1α expansion medium, 6.5% ± 1.2% of the initially plated $Lin^-/34^+/DR^{dim}$ cells could initiate hematopoiesis in one or more secondary cultures, indicating net conservation of LTC-IC. In contrast to the Flt3-L/SCF/IL-7 expansion medium cultures, only 13.9% ± 8.5% of the single $Lin^-/34^+/DR^{dim}$ gave rise to more than 1 LTC-IC. The overall LTC-IC frequency of 8.6% ± 2.1% at week 4 was only 1.2-fold higher than that measured in freshly sorted $Lin^-/34^+/DR^{dim}$ cells (Table 3). Thus, lack of long-term expansion is caused by poor proliferation, and not poor net-conservation. Further, only 4 of 528 $Lin^-/34^+/DR^{dim}$ cells cultured for 4 weeks in IL-3/MIP-1α expansion medium cultures could generate one or more NK-IC in secondary cultures. Finally, no ML-IC could be detected under these conditions.

DISCUSSION

In this report, we describe a novel in vitro assay that can enumerate very primitive human progenitors capable of generating multiple secondary LTC-IC and NK-IC. We term this primitive progenitor a Myeloid-Lymphoid Initiating Cell, or ML-IC. A similar progenitor, termed $LTC\text{-}IC_{ML}$, capable of initiating secondary long-term myeloid cultures as well as pre-B cell cultures, has been characterized in murine bone marrow.[32] As for the human ML-IC, which is five to ten times less frequent than human LTC-IC, murine $LTC\text{-}IC_{ML}$ are 15 times less frequent than murine LTC-IC. A number of studies have described assays that allow human progenitors to differentiate into mature myeloid as well as NK and B- and T-lymphoid cells.[20-22,33-36] The assay described here can assess a possibly even more primitive human bone marrow cell capable of generating multiple secondary progenitors that can reinitiate both myeloid and lymphoid long-term cultures. We believe that this characteristic places the ML-IC ontogenetically very close to the HSC. We have, however, not proven that ML-IC can self-replicate. Therefore, we have not yet shown that ML-IC are human HSC. This will require retroviral tagging. The frequency of ML-ICs is still 10-fold higher than that of SCID-repopulating cells (SRCs), as defined by Larochelle et al.[10] Thus, ML-IC may still contain a population of cells that is less primitive than SRC. Alternatively, differences in frequency may be related to the relative inefficient seeding efficiency of human cells in vivo, leading to underestimation of the absolute number of engrafting human cells. Studies are currently underway to address the relation between SRC and ML-IC.

What are the disadvantages and advantages of the ML-IC assay over the SRC assay? A disadvantage of the ML-IC assay is the cumbersome nature of the technically demanding long-term culture system. A second disadvantage of the ML-IC assay is that "engraftment" per se—a characteristic ascribed to stem cells—cannot be shown. The first advantage of the ML-IC assay is that it can provide information concerning the multipotentiality of a single-sorted CD34 cell. Similar information can be obtained from experiments in which retrovirally tagged human progenitors are transplanted in immunodeficient mice.

Another advantage of the ML-IC assay is that it provides insight into the fate of single LTC-IC and NK-IC cells in ex vivo cultures. We show, for instance, that differences in LTC-IC or NK-IC expansion in different culture systems can be attributed to variable losses in progenitor conservation or progenitor proliferation. These conclusions are in part indirect because progenitor differentiation can occur in the presence or absence of progenitor proliferation. Indeed, an LTC-IC can remain quiescent for the duration of the expansion culture and retain its LTC-IC function. Alternatively, an LTC-IC can die or remain quiescent, but differentiate. Finally, an LTC-IC can proliferate symmetrically or asymmetrically. Each of these outcomes can be investigated using the ML-IC assay.

In Fig 3 we included data previously described by our group,[37] using stroma-conditioned medium supplemented with IL-3 + MIP-1α (SCM/IL-3/MIP-1α). By comparing the expansion systems, we show that presence of AFT024 feeders in the culture is important for net conservation of LTC-IC (and NK-IC). Net conservation of 80% to 130% of LTC-IC and >100% of NK-IC is observed in AFT024-based cultures, and this irrespective of the expansion medium, whereas <30% of LTC-IC is conserved in SCM + IL-3 + MIP-1α (Fig 3). Thus, factor(s) produced by AFT024 may be responsible for the net conservation of LTC-IC. Moore et al have shown that AFT024 feeders support the in vitro maintenance of competitive repopulating murine stem cells for at least 7 weeks.[23] It is thought that expression of one or more novel factors in AFT024 cells is responsible for the improved maintenance of repopulating murine stem cells and possibly for the improved conservation of LTC-IC. One such factor may be the delta-like (dlk) protein/preadipocyte factor-1 (pref-1), which is produced by AFT024

Table 3. LTC-IC Expansion in AFT024/MIP-1α/IL-3 Cultures Is Due to LTC-IC Net-Conservation but Only Limited Net-Proliferation

		LTC-IC at Week 4						
			No Proliferation		Proliferation			
Exp.	Day 0 LTC-IC (%)	Conserved LTC-IC	1 LTC-IC	2 LTC-IC	3 LTC-IC	4-6 LTC-IC	Total LTC-IC	Fold Expansion
1	8.1%	11/132* (8.3%)	7/11†	2/11	2/11	0/11	17/132‡ (12.9%)	1.6
2	5.9%	8/132* (6.1%)	8/8†	0/8	0/8	0/8	8/132‡ (6.1%)	1.0
3	8.0%	15/180* (8.3%)	10/15†	2/15	2/15	1§/15	25/180‡ (13.9%)	1.7
4	4.0%	4/187* (2.1%)	4/4†	0/4	0/4	0/4	4/187‡ (2.1%)	0.5
5	12.9%	14/176* (7.9%)	14/14†	0/14	0/14	0/14	14/176* (7.9%)	0.6

*Number of single-sorted $Lin^-/34^+/DR^{dim}$ cells that generated at least 1 secondary LTC-IC per initially plated cell.

†Number of LTC-IC (per conserved LTC-IC) that did not proliferate (only 1 secondary LTC-IC).

‡Total number of secondary LTC-IC per single sorted $Lin^-/34^+/DR^{dim}$ cell.

§Progeny from initially plated single cells was divided into 6 secondary plates. This particular progenitor generated 5 secondary LTC-IC.

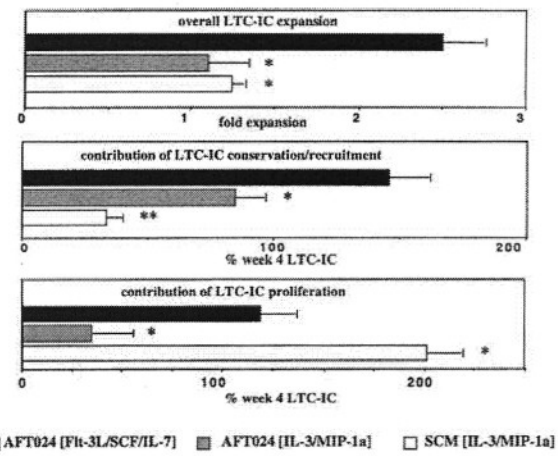

Fig 3. Contribution of proliferation and conservation to overall LTC-IC expansion. Single cell assays were performed as described in Methods and Fig 2 legend. In addition, we used data from a study previously published by our group in which LTC-IC were assessed in a single cell assay based on bone marrow (BM) stroma–conditioned media supplemented with IL-3 and MIP-1α in the absence of a stromal feeder.[37] Significantly greater LTC-IC expansion is seen in AFT024/Flt3-L/SCF/IL-7 cultures than in AFT024/IL-3/MIP-1α cultures or SCM/AFT024/IL-3/MIP-1α cultures. This is a result of both increased proliferation and conservation when compared with AFT024/IL-3/MIP-1α cultures. The equivalent LTC-IC expansion seen in AFT024/IL-3/MIP-1α and SCM/IL-3/MIP-1α cultures is caused by different mechanisms: extensive self-renewal (80.5% ± 7.0%) of only 33.9% ± 6.0% conserved LTC-IC in SCM/IL-3/MIP-1α cultures and minimal self-renewal (13.9% ± 8.6%) of 84.8% ± 11.5% conserved LTC-IC in AFT024/IL-3/MIP-1α cultures. *$P < 0.05$; **$P < 0.01$.

cells, but not by nonsupportive feeders.[38] When dlk/pref-1 is introduced in nonsupportive feeders, ex vivo maintenance of murine repopulating stem cells can in part be restored.[38] Dlk/pref-1 is related to the family of Notch ligands.[39-41] Expression of dlk/pref-1 is downregulated in embryonic tissues during differentiation, and its overexpression prevents terminal differentiation, similar to what is seen with Notch-ligand/Notch-receptor interactions.[39-41] However, dlk/pref-1 does not contain the delta/serrate/lag-2 (DSL)-domain, thought to be required for the binding of Notch ligands to their receptors.[40] Alternatively, AFT024 feeders may produce other novel factors responsible for the improved conservation of primitive progenitors.[42,43]

These same studies also show that more than 80% of LTC-IC proliferated in SCM/IL-3/MIP-1α cultures, but less than 20% in AFT024/IL-3/MIP-1α cultures (Fig 3). This could be a result of contact-mediated proliferation inhibition of progenitors cultured in contact with AFT024 cells because we have previously shown that contact between progenitors and stromal components, such as fibronectin, inhibits proliferation.[44,45] We cannot exclude that some factors present in human marrow–conditioned medium, but not in long-term bone marrow culture (LTBMC) medium used in AFT024/IL-3/MIP-1α, may contribute to the increased proliferation in SCM/IL-3/MIP-1α conditions. Of interest, approximately 50% of the conserved LTC-IC and NK-IC proliferated in AFT024/Flt3-L/SCF/IL-7, the only culture condition allowing significant expansion of LTC-IC and NK-IC. Thus, conservation as well as proliferation is necessary for an effective expansion of primitive progenitors.

In comparing the different culture conditions (AFT024/Flt3-L/SCF/IL-7 and AFT024/IL-3/MIP-1α), we used different culture conditions in the readout system to assess the number of LTC-IC that was present. One could argue that differences in the perceived LTC-IC proliferation and conservation are secondary to the differences in the readout phase, but not the expansion culture phase of the assay. We have, however, previously shown that that assessment of LTC-IC maintenance is similar when measured on AFT024 feeders with or without IL-3/MIP-1α.[30] We show here that the number of LTC-IC measured in single cell assays on AFT024 feeders is equivalent when done in Flt3-L/SCF/IL-7 medium (6.6% ± 0.9% LTC-IC frequency) or in IL-3/MIP-1α medium (7.8% ± 1.5% LTC-IC frequency). Therefore, we believe that differences seen between AFT024/Flt3-L/SCF/IL-7 and AFT024/IL-3/MIP-1α expansion cultures are not a result of differences in our ability to assess LTC-IC in the readout phase of the assay.

In conclusion, we developed an assay that provides indirect insight in the fate of single primitive LTC-IC and NK-IC. Although the information on progenitor proliferation and conservation is in part indirect, insight in the role of net-conservation and net-proliferation of progenitors in a given culture system can be used to guide the investigator in the design of improved expansion systems that are adjusted to increase progenitor conservation and/or proliferation. In addition, this assay allows enumeration of a very primitive progenitor capable of generating multiple secondary LTC-IC and NK-IC, which we termed an ML-IC. This cell is ontogenetically closely related to the HSC. The ML-IC assay can show that a single cell can generate multiple progenitors with multilineage potential. Studies are ongoing to determine what the relationship is between ML-IC and SRC.

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Single Adult Human CD34$^+$/Lin$^-$/CD38$^-$ Progenitors Give Rise to Natural Killer Cells, B-Lineage Cells, Dendritic Cells, and Myeloid Cells

By Jeffrey S. Miller, Valarie McCullar, Michael Punzel, Ihor R. Lemischka, and Kateri A. Moore

Marrow stromal cultures support adult CD34$^+$/Lin$^-$/HLA-DR$^-$ or CD34$^+$/Lin$^-$/CD38$^-$ cell differentiation into natural killer (NK) or myeloid cells, but unlike committed lymphoid progenitors (CD34$^+$/Lin$^-$/CD45RA$^+$/CD10$^+$), no B cells are generated. We tested whether different microenvironments could establish a developmental link between the NK and B-cell lineages. Progenitors were cultured in limiting dilutions with interleukin-7 (IL-7), flt3 ligand (FL), c-kit ligand (KL), IL-3, IL-2, and AFT024, a murine fetal liver line, which supports culture of transplantable murine stem cells. NK cells, CD10$^+$/CD19$^+$ B-lineage cells and dendritic cells (DC) developed from the same starting population and IL-7, FL, and KL were required in this process. Single cell deposition of 3,872 CD34$^+$/Lin$^-$/CD38$^-$ cells onto AFT024 with IL-7, FL, KL, IL-2, and IL-3 showed that a one time addition of IL-3 at culture initiation was essential for multilineage differentiation from single cells. Single and double lineage progeny were frequently detected, but more importantly, 2% of single cells could give rise to at least three lineages (NK cells, B-lineage cells, and DC or myeloid cells) providing direct evidence that NK and B-lineage differentiation derive from a common lymphomyeloid hematopoietic progenitor under the same conditions. This study provides new insights into the role of the microenvironment niche, which governs the earliest events in lymphoid development.

THERE HAS BEEN MUCH interest in mechanisms by which the marrow microenvironment governs lymphoid differentiation through its supportive extracellular matrix, cell surface ligands, and production of soluble cytokines and proteoglycans. We have shown that culture of CD34$^+$/Lin$^-$/DR$^-$ cells from adult marrow will induce differentiation into phenotypic and functional natural killer (NK) cells if progenitors are grown in direct contact with normal allogeneic stroma and interleukin-2 (IL-2).[1] In mice, the ability of stroma to induce differentiation is, at least in part, regulated by the transcription factor interferon-regulatory factor-1 (IRF-1).[2] IRF-1 knockout mice exhibit a severe NK deficiency, which is mediated by failure of transcriptional regulation of IL-15. In human studies, IL-15 made by stroma and monocytes plays a role in NK development and survival by interaction with components of the IL-2 receptor.[3,4] The production of IL-15 by monocytes may explain why NK differentiation can occur in the absence of exogenous IL-15 or stroma.[5] However, stroma still provides other factors to induce the most primitive adult marrow progenitors to develop along the NK lineage. The requirement for direct contact with intact stroma distinguishes this population from more committed progenitors (CD34$^+$/CD7$^+$), which do not require direct contact with stroma for differentiation.[6]

From the Department of Medicine, University of Minnesota Cancer Center, Minneapolis, MN; and the Department of Molecular Biology, Princeton University, Princeton, NJ.

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Address reprint requests to Jeffrey S. Miller, MD, University of Minnesota Cancer Center, Box 806, Division of Hematology, Oncology and Transplantation, Harvard St at East River Rd, Minneapolis, MN 55455; e-mail: mille011@tc.umn.edu.

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Culture of human primitive progenitors with IL-2 and stroma results in terminal differentiation of NK cells. Progeny of long-term NK cultures cannot initiate secondary long-term NK cultures or support other lymphoid or myeloid lineages. The role of other defined cytokines in NK cell commitment and differentiation was assessed by studies using CD34$^+$/CD33$^-$ progenitors after 14-day culture in IL-3 and macrophage inflammatory protein (MIP)-1α, which are highly clonogenic for myeloid long-term culture-initiating cells (LTC-IC).[7,8] CD34$^+$/CD33$^-$ cells from these cultures do not differentiate into NK cells when cultured with IL-2 and stroma until IL-7 and c-kit ligand (KL) were added.[9] We have also been exploring the molecular events, which may occur using in vitro culture. Fresh double sorted CD34$^+$/Lin$^-$/DR$^-$ cells do not express transcripts for RAG-2, CD3γ, CD3δ, or CD3ζ. However, culture with stromal-conditioned media, IL-7, FL, KL, IL-2, and IL-3 not only induced CD3γ, CD3δ, or CD3ζ, but also RAG-2 suggesting possible early T-or B-cell development.[10] The role of FL in lymphopoiesis is further highlighted by defects identified in flt3-deficient mice,[11] and recently, the overlapping and distinct roles of FL and KL in hematopoiesis have been reviewed.[12]

There are many similarities between NK cell and B-cell development. The importance of stroma for B-lymphoid progenitor differentiation has been described for several lymphoid culture systems and is the basis for the Whitlock-Witte culture.[13] The control of B lymphopoiesis requires survival, proliferation, and differentiation signals from the bone marrow microenvironment.[13-15] In the absence of stroma, immature B-cell progenitors rapidly die by apoptosis.[16] Normal B-cell progenitors adhere to stroma through $\alpha4\beta1$ integrin.[17] Disruption of this adhesive interaction results in decreased B-cell differentiation. Normal human B-cell progenitors cultured while physically separated from stroma by a microporous membrane lead to decreased proliferation of CD34$^+$/CD10$^+$ pro-B cells suggesting the importance of direct stromal contact.[18] Similar experiments with B-cell acute lymphocytic leukemia demonstrated decreased cell survival by an apoptotic mechanism when stroma contact was prohibited.[19] Moreover, maximal proliferation of murine pre-B cells in a M2-10B4–dependent culture occurs through RGD-dependent binding of B-cell precursors to fibronectin, which is lost when differentiation occurs.[20]

In this report, we questioned whether an alternate microenvironment could induce human adult marrow progenitors to

ity is determined within 24 hours of staining using a fluorescent microscope.

TdT determination by PCR. After determining the phenotypic presence of CD19 positive cells by flow cytometry, the remaining progeny of single cell cultures were used for determination of TdT by reverse transcriptase-polymerase chain reaction (RT-PCR). Total mRNA was extracted using RNeasy spin columns according to the manufacturer's recommendations (Qiagen, Santa Clarita, CA). Reverse transcription was performed as previously described.[10] Briefly, samples were subjected to 40 cycles of denaturation at 95°C for 20 seconds, annealing at 55°C for 15 seconds, and extension at 72°C for 1 minute in a Perkin Elmer 480 thermal cycler (Applied Biosystems, Foster City, CA). Oligonucleotide primer sequences were: TdT 5 primer: 5 -ACACGAATGCAGAAAGCAGGA-3 ; TdT 3 primer: 5 -AGGCAACCTGAGCTTTTCAAA-3 (provided by Dr T. LeBien); β-actin 5 primer: 5 -TACCTCATGAAGATCCTCA-3 ; β-actin 3 primer: 5 -TTCGTGGATGCCACAGGAC-3 . Amplified products were size separated on 1.5% agarose gels and transferred to Hybond N^+ nucleic acid transfer membranes (Amersham, Arlington Heights, IL). Probes were labeled with ^{32}P-deoxyadenosine triphosphate (dATP) using a TdT 3 -end labeling kit (Boehringer Mannheim, Indianapolis, IN) using probe sequences: TdT 5 -ACACGAATGCAGAAAGCAGGA-3 (provided by Dr T. LeBien); β-actin 5 -CCATCTCTT-GCTCGAAGTC-3 .

Statistics. Results of experimental points obtained from multiple experiments were reported as mean ± 1 standard error of the mean (SEM). Significance levels were determined by two sided Student's *t*-test analysis.

RESULTS

Although primitive $CD34^+/Lin^-/DR^-$ progenitors can be induced to differentiate along the NK cell lineage when cultured with IL-2 in contact with primary adult marrow allogeneic stroma, these cultures result in terminal differentiation and do not support B-lineage cells or other lineages. Therefore, we designed experiments to assess stromal feeder cell lines, which may support differentiation along the NK and B-cell lineage to establish a link between these two lymphoid lineages where differentiation is known to occur in the marrow microenvironment. $CD34^+/Lin^-/DR^-$ cells were isolated from adult marrow and plated with multiple cytokines at culture initiation (IL-7, FL, KL, and IL-2) in the absence of stroma or with two murine feeder cell lines, M2-10B4 and AFT024 (n = 3). Analysis of progenitors cultured without stroma showed little growth and complete absence of NK cell or B-cell progeny. M2-10B4, which supports proliferation of mature NK cells and primitive myeloid cells,[21,33] inefficiently gave rise to NK cell progeny (36% of wells positive at 1,200 cells/well) and no B-lineage cells could be identified. In contrast, coculture of $CD34^+/Lin^-/DR^-$ cells on AFT024 in the presence of IL-7, FL, KL only at culture initiation and IL-2 throughout culture resulted in both NK and B-cell progeny from the same wells (Table 1). Culture of a population of lymphoid-committed cells already expressing one of the lymphoid antigens ($CD34^+/Lin^+$) also gave rise to both NK and B-lineage cell progeny (data not shown). In contrast to the committed $CD34^+/Lin^+$ population where lymphoid progeny were identified by 14 days, differentiation of the more primitive $CD34^+/Lin^-/DR^-$ population required longer (>28 days) culture intervals.

B-lineage cells from the AFT024 cultures were CD19 and CD10 positive and were found to have very low forward and side scatter, significantly smaller than NK cells within the same cultured population (Fig 1). Phenotype analysis showed that the B-lineage cells were negative for CD34 (n = 20), IgM (n = 8), mu (n = 30), kappa light chains (n = 20), and lambda light chains (n = 20), irrespective of the cytokine combination used. Small numbers of $CD19^+$ B-lineage cells were positive for CD20 (18% ± 5.5%, n = 6; P = .005) and CD21 (14% ± 2.9%, n = 13; P = .009) compared with isotype controls (4.3% ± 1.5%). Given this immature phenotype, cells were analyzed further for TdT in situ fluorescent staining. $CD56^+$ NK cells were essentially negative (<2%) for characteristic nuclear TdT staining by fluorescence microscopy (n = 4). In contrast, $CD19^+$ B-lineage cells from the same cultured population were strongly positive in 23% to 55% of cells (n = 4).

Table 1. AFT024 Supports Differentiation of $CD34^+/Lin^-/DR^-$ Cells Into Both NK Cell and B-Lineage Cell Progeny When Cultured With IL-7, FL, KL, and IL-2

No. of Starting Cells/Well	Type of Stromal Feeder	Cultures Analyzed	% Wells With NK Cells	% Wells With B-Lineage Cells
1,200	No Stroma	14	0	0
1,200	M2-10B4	14	36	0
1,200	AFT024	14	100	100
400	AFT024	16	100	62

Cytokines added at culture initiation with weekly media changes containing IL-2 alone. Cells were obtained from 3 donors. Positive wells were determined by >0.5% staining above controls for CD56 (NK) or CD19/CD10 (B).

The precise role of the individual cytokines was assessed by sequential deletion of single cytokines from the four-cytokine cocktail (IL-7, FL, KL, IL-2) using sorted $CD34^+/Lin^-/DR^-$ cells. Comparative cell growth was estimated by flow cytometry as the number of events (per minute) from progeny of each well. Omitting IL-2 from the four-cytokine combination significantly decreased total cell proliferation from 14,660 ± 3,197 to 3,561 ± 312 events per minute (n = 22 wells initiated with 330 cells/well from two donors; P = < .001). However, the number of wells positive for NK cells and B-lineage cells was not significantly different, suggesting that IL-2 is not critical for either NK or B-lineage cell differentiation in the presence of IL-7, FL, and KL. IL-7, FL, and KL were then each omitted from the four-cytokine combination. Deletion of IL-7 slightly decreased relative proliferation (P = .082), did not effect the number of wells with NK cell growth, but significantly decreased the number of wells with B-lineage cell progeny (Fig 2). Although elimination of KL significantly affected relative proliferation, there was no difference in the percent of wells positive for either NK or B-lineage cells. In contrast, omitting FL significantly decreased relative proliferation and the frequency of wells with NK and B-lineage cells (Fig 2), suggesting critical roles for FL and IL-7 in lymphoid differentiation on AFT024. Weekly addition of cytokines lead to increased NK and B-lineage cell proliferation (data not shown), therefore weekly cytokines were used in all subsequent experiments.

Having identified a role for IL-7 and FL, experiments were then performed to determine cloning frequency and lineage-specific cell proliferation by sequential addition of IL-7, FL, KL, and IL-2. IL-3 was also added to this analysis to determine if this primitive acting cytokine would increase cloning fre-

differentiate along the B-cell lineage, which is not found when culturing CD34+/Lin−/DR− progenitors on primary adult stromal layers using current conditions and multiple cytokines. Given the heterogeneity of human stroma containing fibroblasts, adipocytes, macrophages, and endothelial cells, we focused on murine stromal cell lines known to support murine primitive cells. M2-10B4 has been characterized for the ability to support human myeloid LTC-IC.[21] More recently, Moore et al[22] have developed a cell line, called AFT024, which is derived from day 14 gestational fetal liver adherent cells immortalized by introduction of a retrovirus containing a temperature-sensitive SV40 T antigen. Culture of as few as 100 highly purified murine marrow or fetal liver stem cells on AFT024 for 4 to 7 weeks resulted in multilineage reconstitution after transplantation into irradiated mice. In vitro, AFT024 induced proliferation of primitive progenitors and expansion of pro-B–cell progenitors suggesting a novel role for AFT024 in expansion and differentiation of murine hematopoietic progenitors. Therefore, we use the murine AFT024 cell line and defined cytokines to study human hematopoiesis in vitro.

MATERIALS AND METHODS

Normal bone marrow. Bone marrow was obtained from normal donors after informed consent using guidelines approved by the Committee on the Use of Human Subjects in Research at the University of Minnesota. Bone marrow mononuclear cells were obtained by Ficoll-Hypaque (specific gravity, 1.077) (Sigma Diagnostics, St Louis, MO) density gradient centrifugation.

Purification of primitive progenitors. Bone marrow mononuclear cells were enriched for CD34+ cells using an avidin-biotin column as recommended by the manufacturer (Cellpro, Bothel, WA). Resultant cells were stained with CD34-biotin (Cellpro) for multicolor sorting, as previously described.[10,23] Fluorescein isothiocyanate (FITC)-conjugated antibodies against CD2, CD3, CD4, CD5, CD7, CD8, CD10, and CD19 were used for the lineage (Lin) cocktail (Becton Dickinson [BD], San Jose, CA). Phycoerythrin (PE)-conjugated anti–HLA-DR (BD) or anti-CD38 (BD) was used and streptavidin SA670 (GIBCO-BRL, Grand Island, NY) as the third fluorescent color. Single CD34+/Lin−/DR− or CD34+/Lin−/CD38− were sorted directly into 96-well plates using the Automatic Cell Deposition Unit of the FACS Star Plus (BD). For single cell deposition experiments, the Automatic Cell Deposition Unit was set up in a low event "through-put" (200 events/second) and single droplet sorting was used instead of packet sorting to insure deposition of single cells.

Stromal cell lines. Murine stromal cell lines were grown to confluency in 96-well plates and then irradiated (2,000 rad) before use. The M2-10B4 cell line was cultured as described.[21,24] The AFT024 was cloned from murine fetal liver using described techniques[25] and supports the ex vivo culture of murine transplantable stem cells.[22] AFT024 was maintained at 33°C in Dulbecco's Modified Eagle's Medium (DMEM) (GIBCO Laboratories, Grand Island, NY) supplemented with 20% fetal calf serum (HyClone Laboratories, Logan, UT) and 50 μmol/L 2-mercaptoethanol (Bio-Rad, Hercules, CA) and subcultured in 96-well plates precoated with 0.1% gelatin (Specialty Media, Lavalette, NJ).

Culture of hematopoietic progenitors. CD34+/Lin−/DR− or CD34+/Lin−/CD38− cells were plated in a 2:1 (vol/vol) mix of DMEM/Ham's F12-based medium without stroma or in direct contact with stromal cell lines as indicated. The DMEM/F12-based medium (DMEM and Ham's F12 were obtained from GIBCO Laboratories), developed to maximize NK cell growth,[26] was supplemented with 24 μmol/L 2-mercaptoethanol, 50 μmol/L ethanolamine, 20 mg/L L-ascorbic acid, 5 μg/L sodium selenite (Na_2SeO_3), 100 U/mL penicillin, 100 U/mL streptomycin (GIBCO), 20% heat inactivated human AB serum (North American Biologicals, Miami, FL) at culture initiation reduced to 10% for subsequent media changes. Progenitors were plated in limiting dilution assays (22 replicates at four dilutions: 1,000 to 1,200, 300 to 400, 100 to 130, and 33 to 45 cells/well) or by single cell deposition in 96-well plates. The cloning frequency of NK, B, and dendritic cell progenitors was determined by immunophenotyping and was calculated as the reciprocal of the concentration of cells that resulted in 37% negative wells using Poisson statistics and the weighted mean method.[27,28] In limiting dilution experiments, multiple wells were analyzed and reported irrespective of cell growth. In single cell deposition experiments, only those wells which exhibited visual cell growth (>100 cells/well) were analyzed further with three-color immunophenotyping, the remaining wells were considered as having no growth. Cultures were maintained in a humidified atmosphere at 37°C and 5% CO_2 and medium was half changed weekly with the indicated cytokines. Cytokines were supplemented as indicated with 1,000 U/mL IL-2 (a gift from Amgen, Thousand Oaks, CA), 10 ng/mL flt3 ligand (FL, a gift from Immunex, Seattle, WA), 20 ng/mL c-kit ligand (KL or stem cell factor, a gift from Amgen), 20 ng/mL IL-7 (R&D Systems, Minneapolis, MN), and 5 ng/mL IL-3 (R&D Systems). Secondary dendritic cell conditions contained 10 ng/mL tumor necrosis factor (TNF) (R&D Systems), 100 ng/mL granulocyte-macrophage colony-stimulating factor (GM-CSF) (Immunex, Seattle, WA), and 5 ng/mL IL-4 (R&D Systems). Cytokines were added weekly or only once at the time of culture initiation as indicated.

Phenotype, cell quantitation, cytotoxicity, and allogeneic mixed lymphocyte reaction. FITC- and PE-coupled control immunoglobulins or specific antibodies directed at CD2, CD3, CD4, CD7, CD8, CD10, CD11c, CD34, CD19, HLA-DR, kappa light chain, lambda light chain, IgM (all from BD), and mu heavy chain (clone ATTC HB57 from T. LeBien, University of Minnesota Cancer Center, Minneapolis, MN) were used to evaluate progeny of long-term cultures. Three-color phenotype analysis was used to determine multilineage progeny of starting progenitors using CD56-PE, CD19-PerCP, and either CD1a-FITC (PharMingen, San Diego, CA), CD14-FITC (BD), or CD15-FITC (BD). Absolute cell numbers were determined by addition of 3×10^5 polystyrene microspheres (Polysciences, Warrington, PA) to the total progeny of a culture well and after gating out debris, absolute cell numbers were calculated using the method described by Pribyl et al.[29,30] The absolute number of cells/well was calculated as: [(total number of beads added/well)/(number of beads collected) × (number of cells in the phenotype gate of interest)]. The relationship between polystyrene microspheres and absolute cell numbers using this technique was linear between 2.1×10^2 cells/well and 3.3×10^5 cells/well. All analyses were performed with a FACSCalibur (BD) and CELLQuest software (BD). Cytotoxicity assays were performed from progeny of single cells in triplicate using the K562 (American Type Culture Collection, Rockville, MD) cell lines in a 4-hour Cr^{51} release assay.[31] Allogeneic mixed lymphocyte reactions were performed as described with modifications.[32] Briefly, 10^5 allogeneic monocyte-depleted mononuclear cells were incubated in round bottom 96-well tissue culture plates with graded doses of stimulators (30,000 to 3,300) irradiated at 3,000 rads. After 5 days, cultures were pulsed with 1 μCi/well of ^{3}H-thymidine (New England Nuclear, Boston, MA) for 18 hours before harvesting and counting.

Terminal deoxynucleotidyl transferase (TdT) determination by in situ staining. Cytospin preparations of sorted CD19+ or CD56+ cells were fixed for 30 minutes in absolute methanol at 4°C immediately before staining. Rabbit anti-TdT (Supertechs, Bethesda, MD) is diluted 1:10 in phosphate-buffered saline (PBS) buffer and 20 μL is applied to each slide for 30 minutes. After washing, an equal amount of a 1:10 dilution of secondary FITC-goat antirabbit (Supertechs) is applied. TdT positiv-

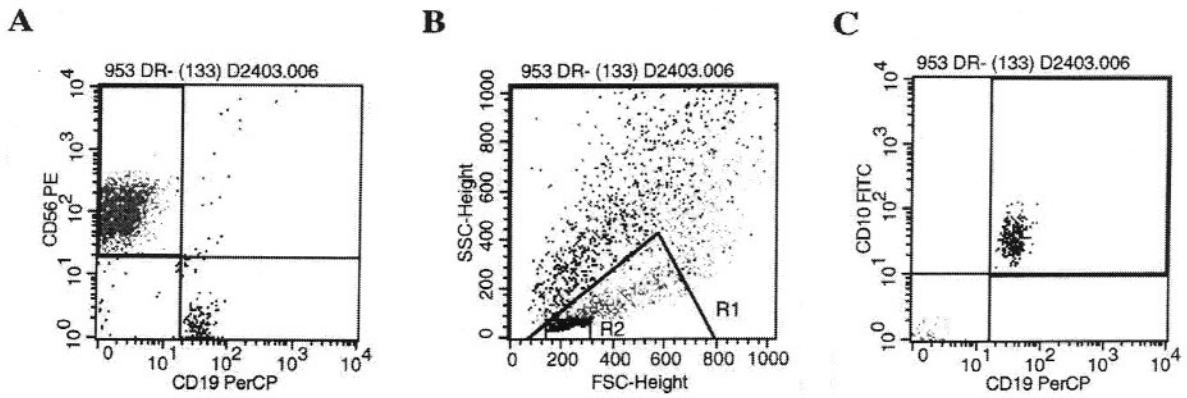

Fig 1. Progeny of AFT024 cultures give rise to NK cells and CD10+/CD19+ B-lineage cells. CD34+/Lin-/DR- cells were cultured in limiting dilutions on AFT024 with IL-7, FL, KL, and IL-2 added at culture initiation. Media was half changed weekly with fresh media supplemented with IL-2 alone. Individual wells were harvested and analyzed by flow cytometry after three-color staining (CD56-PE, CD10-FITC, CD19-PerCP) using appropriate isotype controls. The representative example shown is from a culture well initiated with 130 cells. (A) Shows the two-color analysis of CD56 and CD19. CD19 positive cells were then backgated onto the forward and side scatter plot (B). The CD19+ B-lineage cells (black dots) were significantly smaller than NK cells (medium gray) found within the lymphoid window of the same culture. Backgating to find B-lineage cells in this very small lymphocyte gate (R2) was used as an absolute criteria for all cultures determined positive for B-lineage cells. (C) Shows a representative example of the CD10+/CD19+ B-lineage cells based on the R2 gate.

quency and proliferation or decrease lymphoid capacity, as has been suggested by others.[34] A known number of polystyrene microspheres was added to progeny of each well,[30] allowing precise quantitation of the absolute number of cells by flow cytometry. For these experiments, cytokines were added weekly except for IL-3, which was only added once at culture initiation, because weekly addition of IL-3 lead to cell death from myeloid overgrowth (data not shown). Total cell number (all lineages) was determined for cultures inoculated with 130 $CD34^+/Lin^-/DR^-$ cells on AFT024 with or without sequential addition of cytokines (n = 20 to 38 wells per condition from cells derived from four normal donors). After 35 to 42 days, proliferation was poor with either no cytokines (653 ± 29 cells/well) or IL-7 alone (512 ± 21 cells/well). However, addition of IL-7 + FL (2,717 ± 407 cells/well), IL-7 + FL + KL (21,454 ± 2,390 cells/well), IL-7 + FL + KL + IL-2 (59,595 ± 10,612 cells/well), and IL-7 + FL + KL + IL-2 + IL-3 (108,380 ± 11,712 cells/well) lead to a significant increase in total cell

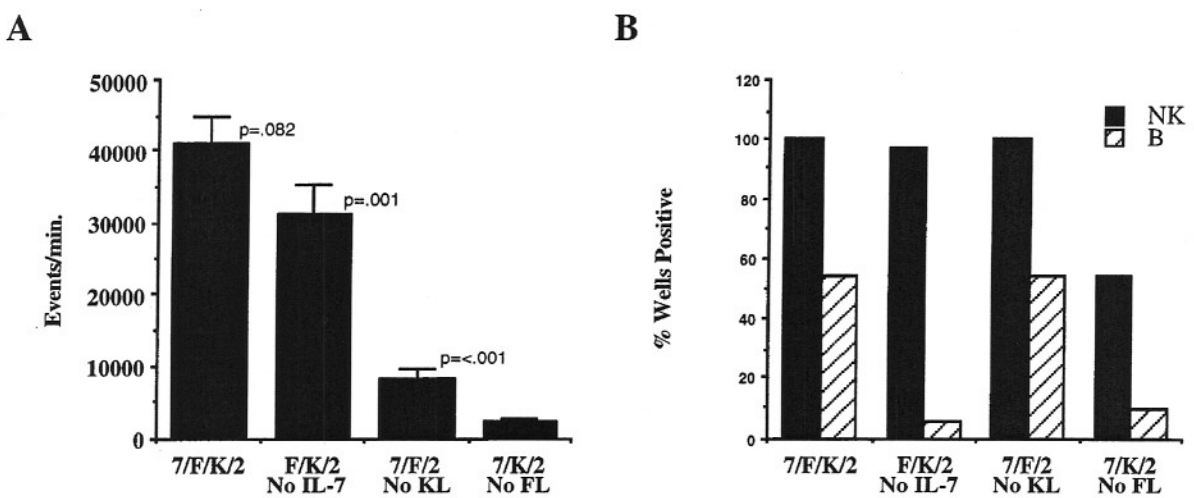

Fig 2. Proliferation and differentiation in AFT024 cultures depends on addition of defined cytokines. CD34+/Lin-/DR- cells (1,000 cells/well) were cultured on AFT024 in 96-well plates with the cytokine combinations indicated. Cytokines were added only once at culture initiation and weekly half media changes contained fresh IL-2 alone. Abbreviations for cytokines in all figures are as follows: 7, IL-7; F, FL (Flt3 ligand); K, KL (c-kit ligand); 2, IL-2; 3, IL-3. After 28 to 35 days in culture, 42 wells per condition from cells derived from three donors were analyzed for proliferation and presence or absence of NK and B-lineage cells. (A) Relative proliferation was determined by flow cytometry as the number of events analyzed per minute. Data are the mean ± SEM for replicate wells analyzed in parallel with the total contents of a harvested well analyzed in a constant volume of approximately 180 μL. The *P* values shown are comparisons between adjacent cytokine combinations. (B) The percentage of positive wells (of 42 wells analyzed per condition) is shown for NK cells (black bars) and B-lineage cells (hatched bars) for each cytokine combination.

proliferation with each additional cytokine ($P = < .03$ for each added cytokine).

Based on phenotype studies, a proportion of cells derived from AFT024 cultures were neither NK or B-lineage cells, suggesting the presence of other lineages. Wright-Giemsa staining showed the presence of myeloid cells (from promyelocyte to neutrophils), monocyte/macrophage cells, and cells with abundant cytoplasmic projections distinct from other cells. Sorting of $CD15^+$ cells from cultures and subsequent cytospin staining showed that all cells were myeloperoxidase positive, further verifying their myeloid origin. Sorting of $CD14^+$ cells from a 4-week culture showed mixed morphologic monocytes, macrophages, and some myeloid cells. $CD14^+$ cells from cultures greater than 8 weeks old showed only macrophages that were myeloperoxidase negative. Sorting $CD1a^+$ cells identified cells with cytoplasmic projections morphologically consistent with dendritic cells (DC). These cells were $CD15^-$ and $CD14^{-/dim+}$. Transfer of progeny from 5 week AFT024 culture into media containing GM-CSF, TNF, and IL-4 further enriched for a population of cells, which were $CD1a^+$, $CD11c^+$, $HLA\text{-}DR^+$, $CD4^+$, $CD15^-$, and $CD14^-$ consistent with the phenotype of cultured DC.[32,35] Cultured cells exhibited characteristic DC function, as primary progeny of AFT024 cultures or progeny of secondary cultures supplemented with GM-CSF, TNF, and IL-4 were capable of stimulating allogeneic T cells in mixed lymphocyte reaction (Fig 3). We could not detect any $CD3^+$ T cells or $CD4^+/CD8^+$ T cells in cultured progeny of AFT024 cultures with any of the cytokine combinations tested. Despite the identification of NK, B-lineage, myeloid, and DC, there was always a population of AFT024 cultured progeny, which was negative for any of the antigens tested, raising the possibility that other lineages may be present as well.

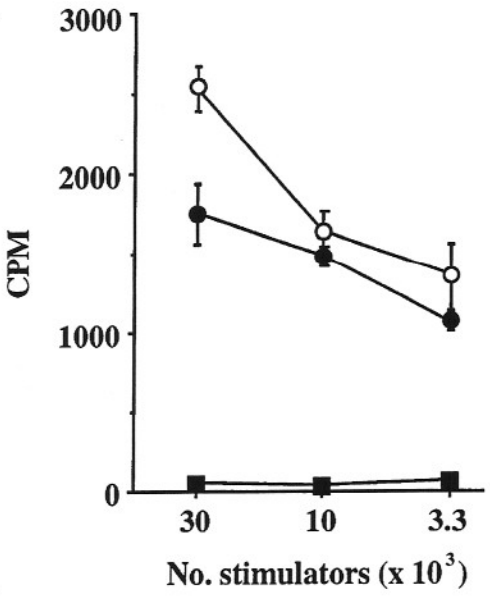

Fig 3. Progeny of AFT024 cultures function to stimulate allogeneic mixed lymphocyte reactions. Monocyte-depleted peripheral blood mononuclear cells (10^5) from the same donor were mixed with irradiated (3,000 rads) autologous $CD14^+$ fresh monocytes (■), 6-week progeny of AFT024 cultures (●), and 5-week progeny of AFT024 cultures transferred for an additional week to media containing TNF, GM-CSF, and IL-4 (○). Proliferation (counts per minute [cpm]) was assessed after 5 days by pulsing for 18 hours with ^{3}H-thymidine and data are presented as the mean ± SEM cpm of triplicate wells from a representative experiment.

The role of cytokines (added weekly except for IL-3) was then evaluated in AFT024 cultures by plating $CD34^+/Lin^-/DR^-$ in limiting dilutions to determine the cloning frequency for NK cells, B-lineage cells, and DC. From these same cultures, the absolute number of cells derived from each positive well could also be calculated. The NK cell cloning frequency of $CD34^+/Lin^-/DR^-$ cells grown without cytokines or with IL-7 alone was less than 0.2% and only slightly increased to 0.5% when IL-7 and FL were combined. However, addition of KL to IL-7 and FL significantly increased ($P = .028$) the NK cloning frequency to over 3% (Fig 4A). Further addition of IL-2 and IL-3 to IL-7, FL, and KL did not change cloning frequency. The role of cytokines on proliferation differed from the role of cytokines on cloning frequency. Proliferation was determined from wells started with 130 $CD34^+/Lin^-/DR^-$ cells cultured for 35 to 42 days (Fig 4B). Even though IL-7, FL, and KL had the greatest impact on cloning frequency, the number of absolute NK cells per well was low (507 ± 100, n = 36). In contrast, addition of IL-2 and further addition of IL-3 at culture initiation significantly increased NK proliferation. The absolute number of NK cells derived from 130 cells when all cytokines were used was 69,354 ± 10,108 NK cells/well (n = 38).

The cloning frequency of CD1a positive DC was similar to that of NK cells when a minimum of IL-7, FL, and KL were added to cultures (Fig 5A). This did not change when IL-2 or IL-3 were added. Although the addition of all cytokines consistently resulted in the highest number of absolute DC (2,546 ± 492, n = 37) derived from 130 $CD34^+/Lin^-/DR^-$ cells, individual cytokine combinations had less influence on proliferation until only IL-7 and FL were added, which resulted in 278 ± 122 DC per positive well (n = 20). B-lineage cell cloning frequency and proliferation was significantly less than either NK cells or DC. Culture of $CD34^+/Lin^-/DR^-$ cells in limiting dilutions on AFT024 with IL-7, FL, and KL (n = 8) resulted in a significantly higher B-lineage cell cloning frequency than any other cytokine combination tested (Fig 5B). Although this same combination induced the highest number of B-lineage cells per well (89 ± 19), given the low overall cloning frequency, the B-lineage cell proliferation did not change significantly with the different cytokine combinations.

Bulk cultures identified conditions optimal for inducing NK cell, B-lineage cell, and DC differentiation and proliferation from adult marrow primitive progenitors. However, we still could not conclude that all of these cell lineages were derived from a single cell. We also questioned whether the role of certain cytokines may be more important at the single cell level rather than in bulk cultures. To answer these questions, experiments were performed by single cell deposition of primitive progenitors from four normal donors onto AFT024 stromal layers using flow cytometry. To maximize outgrowth of primitive progenitors, single cells were sorted $CD34^+/Lin^-/CD38^-$ based on findings that this population may contain a higher frequency of the primitive human progenitors than the $CD34^+/Lin^-/DR^-$ population. Sorting windows were chosen so that the

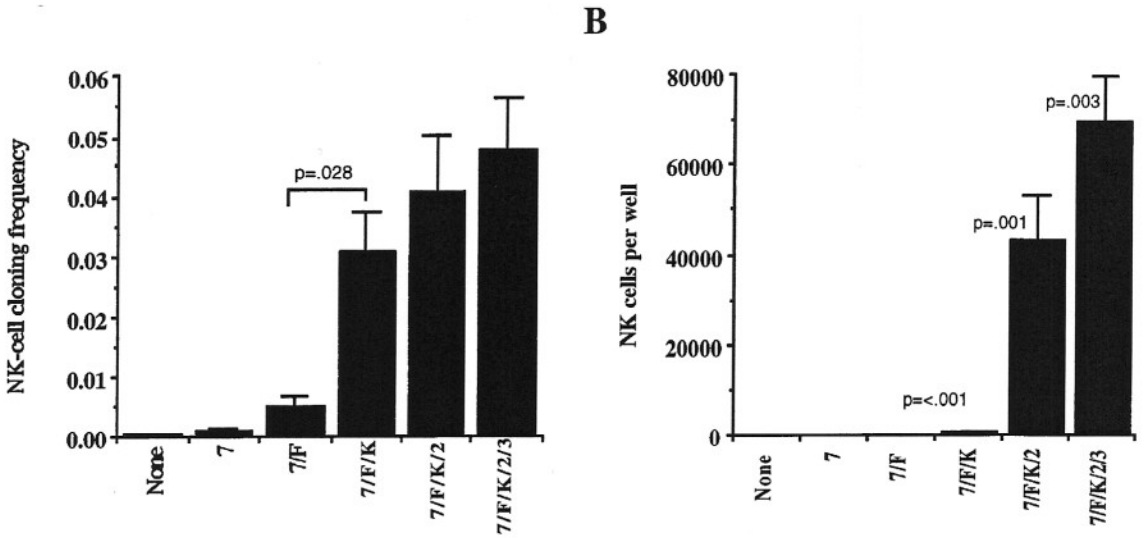

Fig 4. NK cell cloning frequency and absolute NK cell proliferation in AFT024 cultures is dependent on the addition of exogenous cytokines. CD34+/Lin−/DR− cells were plated in limiting dilutions (replicates of 1,200 cells/well, 400 cells/well, 130 cells/well, and 45 cells/well) on AFT024 in 96-well plates with the cytokine combinations indicated. Cultures were maintained with weekly half media changes and fresh cytokines were added weekly except for IL-3, which was only added once at culture initiation. (A) After 35 to 42 days of culture, wells were analyzed using three-color flow cytometry for the presence of CD56+ NK cells to calculate the cloning frequency of initially plated CD34+/Lin−/DR− cells. Cells were gated on viable cells and any well containing greater than 20 absolute CD56+ cells was counted as positive. Each bar represents the mean ± SEM cloning frequency from four donors. (B) The absolute number of NK cells per positive well initiated with 130 CD34+/Lin−/DR− cells is shown for each cytokine combination. Absolute cell counts per harvested well was determined by addition of a known number of polystyrene microspheres to each sample before analysis by flow cytometry as described in Materials and Methods. Each condition represents the mean ± SEM of 20 to 38 individual wells initiated with CD34+/Lin−/DR− cells derived from four donors. *P* values listed are for comparisons between adjacent conditions.

$CD34^+/Lin^-/CD38^-$ population accounted for 2.4% ± 1.2% of total $CD34^+$ cells.

A total of 3,872 single $CD34^+/Lin^-/CD38^-$ cells were sorted onto AFT024 with IL-7, FL, and KL with or without the addition of IL-2, IL-3, or the combination (Table 2). $CD34^+/Lin^-/CD38^-$ cells plated without IL-3 exhibited significantly less single cell growth with 6.3% positive wells versus 27% when IL-3 was included. There was only one positive well of 1,584 plated without IL-3 that gave rise to B-lineage cells compared with 46 of 2,288 wells positive for B-lineage cells when IL-3 was included only at culture initiation. More than 10% of wells with IL-3 at culture initiation gave rise to NK cells and either DC or myeloid cells. Most importantly, 1.5% to 2.0% of single $CD34^+/Lin^-/CD38^-$ cells plated under optimal conditions gave rise to three lineages (NK cells, B-lineage cells, and either $CD1a^+$ DC, $CD15^+$ myeloid cells, or $CD14^+$ monocytes) demonstrating origin from the same cell (Fig 6).

Single-cell proliferation was analyzed using data from Table 2. Exogenous cytokines played a significant role in cell proliferation from single cells (Fig 7A). Although the frequency of single cells giving rise to any progeny was most influenced by initial addition of IL-3, analysis for the absolute number of cells derived from single cells showed contributions to NK cell and DC proliferation from both IL-2 and IL-3 (Fig 7B and C). The absolute number of B-lineage cells in positive wells cultured with IL-7, FL, KL, IL-3, and IL-2 was 190 ± 83.

To further evaluate the B-lineage cells from AFT024 cultures, progeny of single cells were evaluated for TdT mRNA by RT-PCR. Progeny of single $CD34^+/Lin^-/CD38^-$ cells were analyzed by three-color flow cytometry for the presence of NK cells, B-lineage cells, and other lineages. The remaining cells were used for mRNA extraction after identifying the phenotypic presence or absence of $CD19^+$ cells. All of the single cell progeny (n = 9), which were phenotypically positive for B-lineage cells were positive for TdT transcripts. In contrast, in wells where B-lineage cells could not be phenotypically identified, seven of 17 were still positive for TdT. The finding of TdT in 40% of phenotypic B-lineage cell negative populations suggests that the number of wells giving rise to B-lineage cells may be underscored by phenotypic identification alone. Finally, after 42 days of culture with IL-7, FL, KL, IL-3, and IL-2, progeny of single $CD34^+/Lin^-/CD38^-$ cells cocultured with AFT024 were selected based on growth for expansion in 24-well plates with IL-2 alone to obtain enough NK cells for functional and NK cell subset analysis. The 16 populations derived from single $CD34^+/Lin^-/CD38^-$ cells gave rise to an average of 8.3×10^5 cells (range, 3 to 22×10^5) of which 94% ± 1.0% were $CD56^+/CD3^-$, 11% ± 2.0% were $CD56^+/CD2^+$, and 21% ± 5% were $CD56^+/CD7^+$. These NK cells exhibited characteristic function against K562 targets in cytotoxicity assays (Fig 8).

DISCUSSION

We have developed a novel long-term culture assay using the murine fetal stromal cell line, AFT024, and human cytokines (IL-7, FL, KL, IL-2, and IL-3) to induce multilineage lymphoid

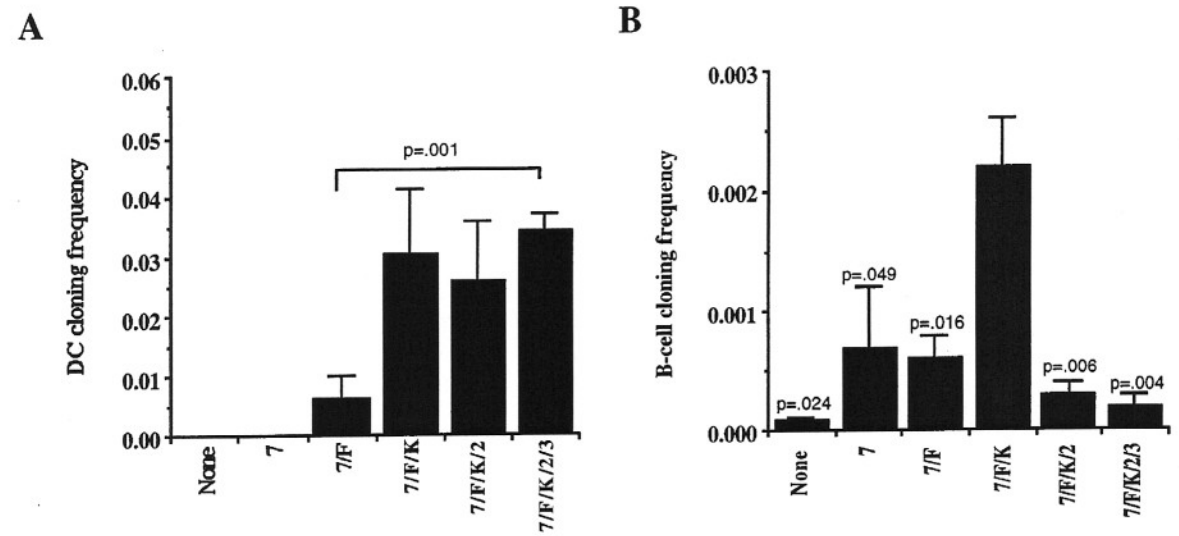

Fig 5. DC and B-lineage cell cloning frequency in AFT024 cultures is dependent on the addition of exogenous cytokines. CD34⁺/Lin⁻/DR⁻ cells were plated in limiting dilutions on AFT024 in 96-well plates with the cytokine combinations indicated for 35 to 42 days. (A) Multiple replicates were analyzed using three-color flow cytometry for the presence of CD1a⁺ cells to calculate the cloning frequency of initially plated CD34⁺/Lin⁻/DR⁻ cells. Cells were gated on viable cells and any well containing greater than 20 absolute CD1a⁺ cells was counted as positive. Each bar represents the mean ± SEM cloning frequency from four donors. (B) Replicates were analyzed for the presence of CD19⁺ cells to calculate the B-lineage cell cloning frequency. Cells were gated on a very low forward and side scatter (Fig 1B designated R2) and any well containing greater than 10 absolute CD19⁺ cells was counted as positive. Each bar represents the mean and SEM cloning frequency from four donors except for the condition using IL-7, FL, and KL (7/F/K) where eight donors were used. All *P* values shown are compared with the frequency using IL-7, FL, and KL.

and myeloid differentiation from adult human marrow progenitors. The multilineage potential of this murine stromal-based system using one culture condition was striking. In addition to inducing NK differentiation more efficiently than found previously with adult human allogeneic stroma,[6] CD10⁺/CD19⁺ B-lineage cells were also generated from the same starting cells. Limiting dilution assays using three-color immunophenotype analysis and absolute quantitation of various cell types, determined the role of cytokines on NK cell, B-lineage cell, and DC cloning frequency and proliferation. Single cell sorting of individual CD34⁺/Lin⁻/CD38⁻ cells verified that not only NK cells and B-lineage cells derive from the same cell, but DC and mixed myeloid cells (granulocytic/monocytic) as well. Finding that most single cells that resulted in B-lineage cell differentia-

Table 2. NK Cell, B-Lineage Cell, Dendritic Cell, and Myeloid Progeny From Single Sorted CD34⁺/Lin⁻/CD38⁻ Cells Cultured on AFT024 With IL-7, FL, KL, ±IL-3 ± IL-2

Lineages Derived From Single Cells*	IL-7, FL, KL† 47/792 (5.9%) Wells Evaluated‡		IL-7, FL, KL + IL-2† 53/792 (6.7%) Wells Evaluated‡		IL-7, FL, KL + IL-3† 399/1408 (28%) Wells Evaluated‡		IL-7, FL, KL + IL-2 + IL-3† 221/880 (25%) Wells Evaluated‡	
	Positive (n)§/Total (n)	% Positive	Positive (n)§/Total (n)	% Positive	Positive (n)§/Total (n)	% Positive	Positive (n)§/Total (n)	% Positive
NK (total)	8/792	1.0	21/792	2.6	172/1,408	12.2	159/880	18.1
DC (total)	1/792	0.1	8/792	1.0	86/880	9.8	59/352	16.7
M (total)	—	—	—	—	104/352	29.5	98/352	27.8
B (total)	1/792	0.1	0/792	0	29/1,408	2.1	17/880	1.9
NK + DM	1/792	0.1	6/792	0.7	94/1,232	7.6	107/704	15.2
NK/B + DM	0/792	0	0/792	0	19/1,232	1.5	14/704	2.0

Abbreviations: NK, CD56⁺ natural killer cell; DC, CD1a⁺, dendritic cell; M, CD15⁺ or CD14⁺ myeloid cell; B, CD19⁺ B-lineage cell; DM, either CD1a⁺, CD15⁺ or CD14⁺.

*Lineage was determined by three-color flow cytometry.

†All cytokines added weekly except for IL-3, which was added only once at culture initiation.

‡Wells determined positive by visual cell growth greater than 100 cells/well were analyzed with a three-color immunophenotype and all other wells were counted as negative.

§The number of single cell progeny positive for each lineage or combination of lineages was determined and expressed over the denominator of the total number of single cells plated. All wells were analyzed for NK and B-lineage cells with third fluorescent stain either for DC (CD1a) or M (CD15 or CD14), explaining the lower denominator for these lineages.

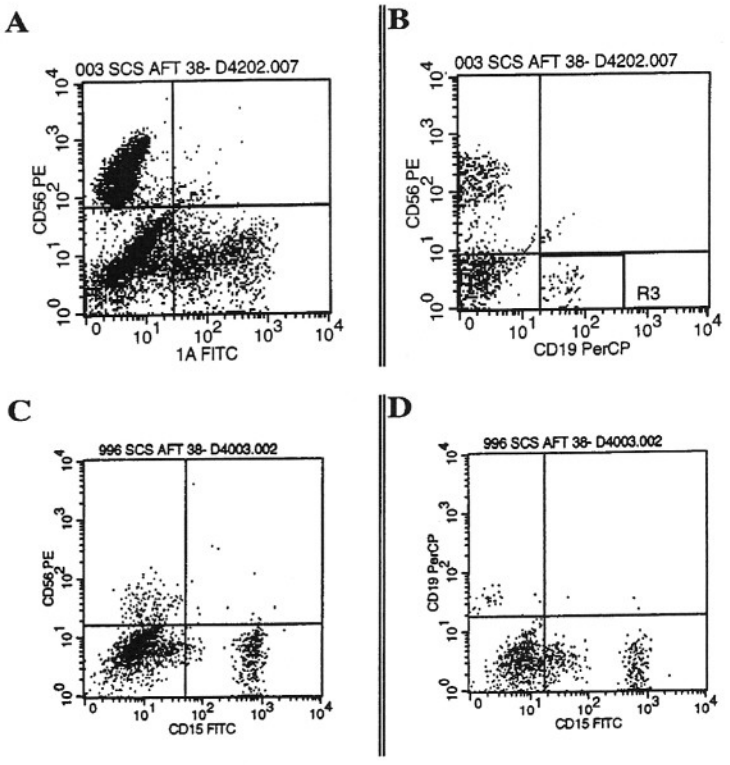

Fig 6. Multiple lineages are present from the cultured progeny of single cells. Single CD34+/Lin-/CD38- cells were cultured on AFT024 for 42 days with IL-7, FL, KL, IL-2, and IL-3 (A and B are from progeny of the same single cell) or with IL-7, FL, KL, and IL-3 (C and D are from progeny of the same single cell). Data are examples of the three-color phenotypes summarized in Table 2 showing the multilineage differentiation, which resulted from single cell cultures gated on all viable cells (panel A and C) or a smaller lymphocyte gate (panel B and D).

tion also resulted in NK cell and DC or myeloid differentiation (at least three lineages from one cell) suggests origin from a very primitive progenitor.

In experiments eliminating single cytokines, the B-lineage cell differentiating capacity of this culture was dependent on FL, IL-7, and stromal ligands in agreement with previous reports in human and murine lymphopoiesis showing a role for one or more of these factors.[36-41] Although the B-lineage cell development from CD34+/Lin-/DR- cells occurred without KL, its addition optimizes growth for readout detection. IL-7, FL, and KL were required to induce development along the NK cell lineage. This is in agreement with Silva et al,[5] who demonstrate that NK differentiation is independent of IL-2 as long as IL-7 is present. The finding that KL potentiates outgrowth fits with the finding of c-kit receptor on lymphoid progenitors and more primitive CD56+bright blood NK cells.[42,43] The ability of AFT024 to induce NK cell, B-lineage cell, and other myeloid lineages from the same cell depends on properties of the murine AFT024 fetal cell line. M2-10B4 produces soluble factors and provides contact-mediated growth promoting ligands to mature NK cells.[24,33] Although M2-10B4 can support primitive myelopoiesis,[21] it poorly supports NK cell differentiation and does not support B-cell development from primitive human progenitors. The capacity of the AFT024 cell line to support lymphoid differentiation may be, in part, related to its developmental fetal origin. Pribyl and LeBien[44] have

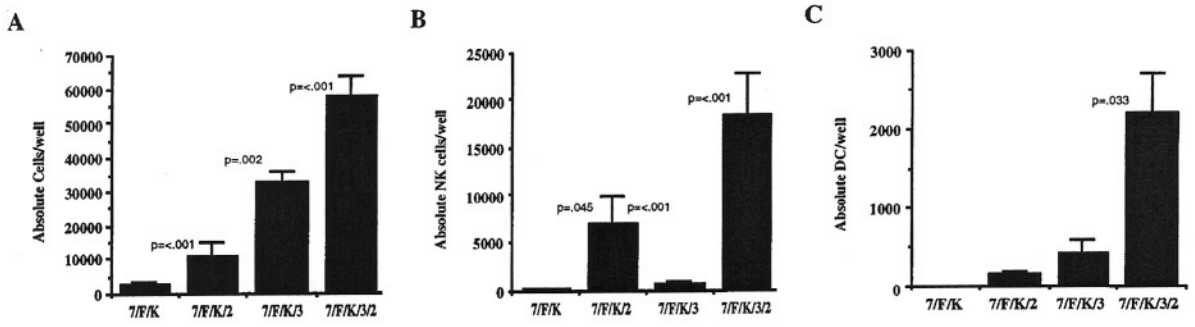

Fig 7. Proliferation of single sorted CD34+/Lin-/CD38- cells is optimal with IL-7, FL, KL, IL-2, and IL-3. The absolute number of total cells (A), NK cells (B), and DC (C) were analyzed from progeny of 2,640 single sorted CD34+/Lin-/CD38- cells summarized in Table 2. Results in (A) are from all wells evaluated per cytokine condition. Results in (B) and (C) are from wells determined positive for NK cell or DC progeny, respectively. The *P* values listed are comparisons between adjacent bars where significant.

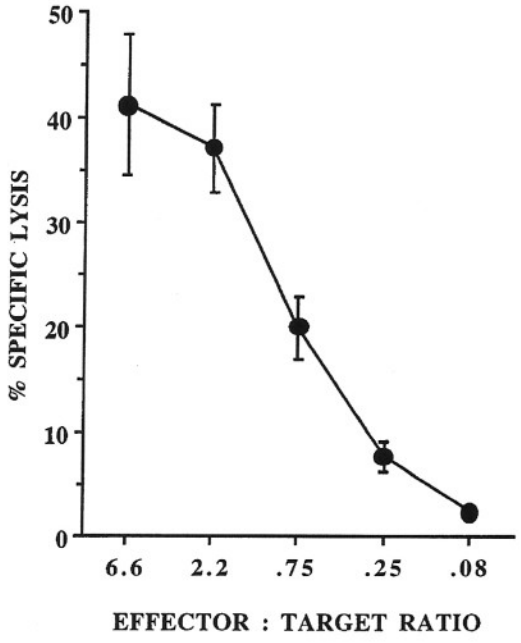

Fig 8. NK cells derived from single cells exhibit cytotoxic activity against K562 targets. Single CD34$^+$/Lin$^-$/CD38$^-$ progenitors were cultured on AFT024 with IL-7, FL, KL, IL-2, and IL-3 (added only at culture initiation) for 42 days and wells with the highest proliferation were transferred to 24-well plates in media containing IL-2 alone for an additional 14 days. Cells were then counted and tested for cytotoxicity against chromium-labeled K562 targets. Results are from 13 NK cell populations derived from progeny of 13 single cells from two normal donors (data represent the mean ± SEM of the average of triplicate wells from each population).

shown that human fetal stroma can differentiate fetal CD34^{++}/Lin$^-$ cells into mu/kappa or mu/lambda expressing B cells and differentiation was IL-7–independent. Alternatively, there may be unique properties of murine stromal cell lines to support B-cell differentiation such as the S17 cell line reported by Rawlings et al[45,46] or by the MS-5 cell line described by Berardi et al.[47] However, the cytokine-independent differentiation observed with S17 and MS-5 is in contrast to results shown here with the AFT024 cell line. In addition to different murine stroma cell lines, results may be explained in part by our use of adult marrow progenitors contrasted to progenitors from cord blood, which may contain more immature stem cells with different cytokine requirements than adult bone marrow.[48]

The B-lineage cells, which developed from adult marrow progenitors, were developmentally blocked at the pro-B cell stage by the absence of surface heavy and light chains and the presence of TdT. The detection of CD20 and CD21 on a small proportion of CD19$^+$ cells may suggest early progression to B-cell maturation with D-J gene rearrangement but the absence of surface IgM shows they are not able to develop into mature B cells.[49,50] The phenotype of B-lineage cells resulting from AFT024 cultures is similar to that shown by Rawlings et al[45] with the exception that less than 3% of their S17 cultured B-lineage cells were TdT positive, while more AFT024 B-lineage cell progeny were positive for TdT by both in situ staining and RT-PCR.

Single-cell experiments differed from bulk cultures in the absolute requirement of a one time addition of 5 ng/mL of IL-3 at culture initiation. Whether this is required for mere survival or proliferation induction is uncertain. The role of early acting cytokines can alter the self-renewal, viability, or proliferation of single CD34$^+$CD38$^-$ cells.[51] For example, in studies of single CD34$^+$/CD38$^-$ progenitors, 60 ng/mL IL-3 in addition to 300 ng/mL of FL and KL were necessary to obtain optimal amplification of myeloid LTC-IC and colony-forming cells (CFC).[52] However, when 10 ng/mL of FL and KL were used, the same concentration of IL-3 decreased LTC-IC expansion and increased CFC expansion, suggesting that the net effect of multiple cytokines is determined not only by the cytokines themselves, but also their relative concentrations. The requirement for IL-3 early in the culture period differs from several murine studies by Ogawa's group demonstrating that IL-3 is inhibitory to B cell, T-cell, and NK cell development.[34,42,53,54] However, the concentration of IL-3 in our cultures was low and distant (4 to 6 weeks) from the readout of lymphoid progeny. In addition, IL-3–induced lymphoid suppression may not be absolute, as the addition of IL-4 in combination with IL-11 or IL-6 reversed the IL-3–induced inhibition on early B-cell development.[55] The sequential, low concentration, one time addition of IL-3 clearly increased NK proliferation, possibly by upregulation of IL-2 receptors by simultaneous exposure to KL, as proposed by Shibuya et al.[56]

The cloning frequency from the single cell experiments was consistently higher than from the limiting dilution assays. This may be explained by competitive interactions within the AFT024 cultures. In addition to the interaction with AFT024, the contribution of human progeny cell interactions induced by AFT024 may be critical to the resultant multilineage lymphoid and myeloid differentiation. Differentiation of DC progeny expressing costimulatory molecules or monocyte progeny secreting IL-15 may interact with B-lineage cells and NK cells, respectively. Similarly, transforming growth factor-β (TGF-β), a potent costimulator of FL-induced DC growth,[57] may be present in culture by activation of latent TGF-β in serum by the extracellular matrix component thrombospondin or by production of TGF-β from developing NK cells.[33] The complexities of these interactions will need further study.

In summary, using the murine fetal liver cell line, AFT024, and defined cytokines, long-term in vitro culture can differentiate single adult human CD34$^+$/Lin$^-$/CD38$^-$ cells into NK cells, B-lineage cells, DC, and myeloid lineages using a single culture condition. Our results are consistent with the NK cell, B-cell and DC differentiation pattern derived from the more committed CD34$^+$/Lin$^-$/CD45RA$^+$/CD10$^+$ adult marrow cell reported by Galy et al.[58] However, the additional myeloid lineage differentiation in AFT024 cultures, not found from CD34$^+$/Lin$^-$/CD45RA$^+$/CD10$^+$ cells, distinguishes our system and suggests multilineage differentiation from a more primitive cell. The AFT024 cell line and IL-7, FL, KL, IL-2, and IL-3 are critical to this process. This in vitro hematopoietic culture gives quantitative information, which will be useful in comparing hematopoietic stem cell sources, and lends itself to easy manipulation of in vitro culture. The ability to efficiently induce differentiation from single primitive cells identifiable from adult marrow will

provide new insights into mechanisms governing the earliest steps in lymphoid differentiation.

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The Microenvironment of AFT024 Cells Maintains Primitive Human Hematopoiesis by Counteracting Contact Mediated Inhibition of Proliferation

MICHAEL PUNZEL[1], PANKAJ GUPTA[2], and CATHERINE M. VERFAILLIE[1]
[1]*Department of Medicine, Division of Hematology and Stem Cell Institute, University of Minnesota, Minneapolis, MN, and* [2]*Department of Veterans Affairs, Medical Center, Minneapolis, MN*

We have previously shown that maintenance of primitive human hematopoietic stem cells is poor when cultured in contact with marrow stromal feeders. However, when separated from stromal contact, human progenitors can be maintained because adhesion mediated proliferation inhibition does not occur. In this study we demonstrate how the murine fetal liver cell line, AFT024, supports primitive human hematopoiesis better in contact cultures compared to primary feeders. We evaluated if better progenitor maintenance in contact with AFT024 cells can be explained by decreased adhesion itself or decreased adhesion mediated inhibition of proliferation. We show that primitive human hematopoietic cells adhered equally well to AFT024 and primary feeders, such as M2-10B4. Further, contact with metabolically inactive AFT024 cells prevented cell cycle progression and decreased maintenance of primitive progenitors to the same extent as contact with M2-10B4 feeders. However, contact with viable AFT024 feeders did not inhibit proliferation, suggesting that AFT024-factors counteract contact mediated inhibition of proliferation. Cytokine production by M2-10B4 and AFT024 cells was similar. Large-size O-sulfated heparan sulfate glycosaminoglycans, known to be important for hematopoietic support, were found only in AFT024-matrix. We hypothesize that these factors may explain, in part, our observations. Finally, we show that more than 100% of primitive myeloid progenitors could be maintained for at least five weeks when cultured in contact with AFT024 feeders in the presence of Interleukin-3 and Macrophage Inflammatory Protein-1α. In conclusion, AFT024 cells produce factor(s), that counteract contact induced growth inhibition of primitive human hematopoietic progenitors, leading to expansion of these cells in contact with the microenvironment.

Keywords. AFT024, human hematopoietic stem cells, proliferation inhibition, O-sulfated heparane sulfate glycosaminoglycans

INTRODUCTION

A number of human and murine cell lines, including, MS-5 (1–3), M2-10B4 (4, 5), SYS-1 (6–8), S17 (9–11), NIH3T3 (5), and FBMD-1 (12), have been described that support to varying degrees immature human hematopoietic progenitors *in vitro*. Moore et al. showed that the murine fetal liver cell

Received 23 November 2001; accepted 19 June 2002.
Address correspondence to Michael Punzel, MD, Medizinische Poliklinik, University of Heidelberg, Hospital Str. 3, 69115 Heidelberg, Germany. E-mail: michael_punzel@med.uni-heidelberg.de
Abbreviations. LTC-IC: Long-Term Culture Initiating Cells; NK-IC: Natural Killer Initiating Cells; HS: Heparane Sulfate; CFC: Colony Forming Cells; BSA: Bovine Serum Albumine.

line, AFT024, supports survival of competitive repopulating murine stem cells for up to seven weeks *ex vivo* and may serve as stem cell preserving microenvironment (13). We and others have shown that AFT024 feeders maintain primitive myeloid cells, such as Long-Term Culture Initiating Cells (LTC-IC) for at least five weeks *ex vivo* and also allow multilineage differentiation of single human CD34+/CD38− bone marrow cells, which can be detected as Myeloid-Lymphoid Initiating Cells (14–18). These studies indicate that AFT024 cells not only support murine stem cells better than heterogenous primary marrow feeders but also support primitive human hematopoiesis.

It is of interest to note that the studies quoted above evaluated progenitor support in stroma contact cultures. We have shown that growth of both primitive and committed human hematopoietic progenitors is inhibited when cultured in contact with primary stromal feeders (5, 19–21). Using a standard thymidine-suicide assay, we found that committed human progenitors, such as Colony-forming Cells (CFC) cultured in contact with primary marrow feeders or extracellular matrix components, such as fibronectin, proliferate only 2–4 days after medium exchange, while CFC cultured in noncontact conditions proliferate continuously between weekly feeding cycles (5, 20). We have shown that inhibition of proliferation is due to integrin mediated upregulation of $p27^{Kip1}$ which prevents cells from entering S-phase of the cell cycle (22). Interestly, addition of Interleukin-3 (IL-3) or Stem Cell Factor (SCF) to assays in which integrins are engaged prevents the integrin mediated inhibition of proliferation. This suggests that certain factors can counteract contact mediated growth inhibition (23). Therefore, we hypothesized that improved progenitor maintenance in AFT024 contact cultures could be due to lack of progenitor adhesion to AFT024 cells. An alternative explanation would be that AFT024 feeders produce one or more factors that counteract contact mediated inhibition of proliferation.

To answer this question and evaluate the effect of adhesion itself on progenitor proliferation, we used our previously described culture system in which feeders had been fixed with glutaraldehyde (20). Glutaraldehyde-fixation inactivates stromal cells metabolically but preserves cell surface expressed adhesive ligands and the extracellular matrix (20). In this study, we demonstrate that one or more factors presented by viable AFT024-feeders, such as large O-sulfated heparan sulfate glycosaminoglycans, counteract contact mediated inhibition of primitive cell growth.

MATERIALS AND METHODS

Cell Sources

Bone marrow was aspirated from the posterior iliac crest from healthy volunteer donors after informed consent using guidelines approved by the Committee on the Use of Human Subjects at the University of Minnesota.

Cell Preparation and Sorting

Mononuclear cells were obtained by Ficoll-Hypaque (Sigma-Diagnostics, St. Louis, MO) centrifugation. CD34+ cells were enriched using MACS™ columns (Miltenyi-Biotec, Bergisch-Gladbach, Germany). To obtain CD34+/HLA-DR− cells, CD34+ enriched cells were labeled with anti-CD34-PE (HPCA2, Becton-Dickinson Immunocytometry Systems, [BDIS], San Jose, CA) and anti-HLA-DR-FITC antibodies (BDIS). To obtain CD34+/HLA-DR−/CD2−/CD7− cells, CD34+ enriched cells were incubated with anti-CD34-Biotin (clone 12.8; Cellpro Inc., Bothel, WA), anti-HLA-DR-PE (BDIS), anti-CD2-FITC (BDIS), and anti-CD7-FITC (BDIS), followed by Streptavidin-SA670 (Gibco-BRL, Grand Island, NY). Cells were sorted on a FACS Star-Plus flow cytometry system (BDIS) equipped with a CONSORT32 computer using isotype-matched controls (17).

Stromal Feeders

The murine marrow derived cell line M2-10B4, (a kind gift from C. Eaves, Terry Fox Laboratories,

Vancouver, Canada) was maintained in RPMI-1640 (Gibco-BRL) +10% Fetal Calf serum (FCS, Hyclone, UT) (4, 5). The murine fetal liver cell line, AFT024 (a kind gift from I. R. Lemischka, Princeton University, Princeton, NJ) (13) was maintained in DMEM (Gibco) supplemented with 20% FCS, 50 μM 2-Mercaptoethanol (Bio-Rad, Hercules, CA) and maintained at 33°C. M2-10B4 or AFT024 cells were subcultured in 24- or 96-well plates (Costar, Cambridge, MA), grown to confluency and then irradiated at 6000 rads (M2-10B4) or at 2000 rads (AFT024). For AFT024, tissue culture dishes were precoated with 0.1% gelatin (Specialty Media, Lavalette, NJ) before seeding the cells.

Glutaraldehyde fixation. After irradiation all mediums were replaced with a 2% glutaric acid dialdehyde solution (Aldrich Chemical Company Inc., Milwaukee, WI) in Hanks buffer (Gibco) for 5 min. Feeders were washed five times with IMDM (Gibco) and incubated overnight at 37°C in IMDM with 20% FCS. The following day the feeders were washed three more times with IMDM (20).

Culture Media

Long-term culture medium (LTBMC-medium). IMDM (Gibco) with 12.5% FCS, 12.5% horse serum (Terry Fox Laboratories), 2 mmol/L L-glutamine (Gibco), 1000 U/ml penicillin, 100 U/ml streptomycin (Gibco), and 10^{-6} mol/L hydrocortisone.

Lymphoid differentiation medium. DMEM (Gibco) and Ham's F12-medium (Gibco) in a 2:1 (V/V) mix containing 20% heat inactivated human AB serum (North American Biologicals, Miami, FL), 20 mg/ml ascorbic acid (Gibco), 50 μmol selenium selenite (Gibco), 25 μmol 2-ME (Biorad), 50 μmol ethanolamine (Gibco), and 1000 U/ml Interleukin-2 (Amgen, Thousand Oaks, CA). At weekly intervals media half change was done using 10% instead of 20% human AB serum (18).

Artificial conditioned medium. We have previously identified cytokines and extracellular matrix components in medium conditioned by bone marrow stroma or M2-10B4 feeders that are required for LTC-IC support (24–26). Based on this information, we generated so called "artificial conditioned medium": LTBMC-medium supplemented with human cytokines at concentrations found in supernatants of human bone marrow stroma (10 pg/ml GM-CSF [Immunex Corp., Seattle, WA], 250 pg/ml G-CSF [Amgen], 200 pg/ml SCF [R&D Systems Inc, Minneapolis, MN], 50 pg/ml LIF [R&D systems], 200 pg/ml MIP-1α [R&D systems], 1 ng/ml IL-6 [a kind gift from Dr. G. Wong, Genetics Institute, Boston, MA]) as well as O-sulfated glycosaminoglycans (10 μg/ml N-desulfated, N-acetylated-Heparin [Seikagaku America Inc., Rockville, MD]). We added 100 ng/ml MIP-1α (R&D systems) and 5 ng/ml IL-3 to the medium.

LTC-IC Maintenance Cultures

Cultures in contact with viable feeders. 10,000 CD34+/HLA-DR− cells were plated on irradiated nonfixed M2-10B4 or AFT024 feeders in LTBMC-medium. Cultures were kept in a humidified atmosphere at 37°C and 5% CO_2 and fed weekly. In indicated experiments, cultures were fed 3×/week with LTBMC-medium with 100 ng/ml MIP-1α and 5 ng/ml IL-3.

Cultures in contact with fixed stroma. 10,000 CD34+/HLA-DR− cells were plated on glutaraldehyde fixed M2-10B4 or AFT024 feeders for five weeks in artificial conditioned medium with 100 ng/ml MIP-1α and 5 ng/ml IL-3 in a humidified atmosphere at 37°C and 5% CO_2. Cultures were kept in a humidified atmosphere at 37°C and 5% CO_2 and fed 3×/week with fresh medium.

Cultures without stroma. 24-well plates were incubated with PBS with 1% Bovine serum albumine (BSA) (Sigma). After 1 h all medium was removed and wells allowed to dry for 2 h under sterile conditions. 10,000 CD34+/HLA-DR− cells were plated in artificial conditioned medium with 100 ng/ml

MIP-1α and 5 ng/ml IL-3 in the BSA coated wells. Cultures were maintained in a humidified atmosphere at 37°C and 5% CO_2 and fed 3×/week with fresh medium.

NK progenitor cultures. 10,000 CD34+/HLA-DR−/CD2−/CD7− cells were plated on viable M2-10B4 or AFT024 feeders. Cultures were maintained in lymphoid differentiation medium in a humidified atmosphere at 37°C and 5% CO_2. Cultures were fed at weekly intervals. After five weeks, cultures were harvested and cells stained with anti-CD56-PE and anti-CD3-FITC antibodies (BDIS) and analyzed by FACS to determine the presence of $CD56^+$/CD3− NK cells. Cells were also examined for their capability to kill K562 targets (27).

Assessment of the Absolute Number of LTC-IC by Limiting Dilution Assay (LDA)

A total of 5,000 CD34+/HLA-DR− cells (22 replicates per concentration: 150, 50, 15, 5 cells/well) were plated onto irradiated confluent M2-10B4 feeders subcultured in 96-well plates (Costar) and maintained for five weeks in LTBMC-medium. Likewise, week 5 progeny of CD34+/HLA-DR− cells were harvested and replated in LDA on M2-10B4. After five weeks, all LTBMC media were removed and replaced with clonogenic methylcellulose medium containing 1.12% methylcellulose, IMDM (Gibco), 30% FCS, 3 IU/ml erythropoietin (Amgen), and supernatant of the bladder carcinoma cell line 5637 (7.5%). Wells were scored for the presence or absence of secondary CFC between day 12 and day 16. The absolute frequency of LTC-IC present in the different cell populations was calculated as described (21).

Adhesion Assays

Column purified CD34+ cells were plated in 24-well plates (200,000 cells per point) containing either viable or fixed confluent M2-10B4 or AFT024 feeders. After 12–16 hours the nonadherent fraction was removed by three washes using warm IMDM. The adherent fraction was collected by trypsinization. The number of adherent and nonadherent CD34+ cells was determined by counting in a hemocytometer. In control experiments adhesion to BSA or poly-L-lysine (PLL, Sigma) coated plates was measured.

Cell Cycle Analysis

Adherent and nonadherent cells were labeled with anti CD34-FITC [BDIS], washed with PBS/0.3% BSA (Sigma) and fixed with 70% ethanol for 2 h in −20°C. Cells were then washed once with IMDM/50% FCS and twice with PBS containing 0.3% BSA. Cells were permeabilized with 0.25% Triton-X100 for 5 min, washed twice with PBS containing 0.3% BSA, and stained with 125 μl propidiumiodide (50 μg/ml, Sigma) for 30 min at 4°C. The fraction of CD34+ cells in G0/G1-, S- and G2/M phase was assessed by flow cytometry using ModFit software (BDIS). Because of autofluorescence of M2-10B4 cells, adherent CD34+ cells were purified by FACS prior to fixation and permeabilization.

Structural Analysis of Heparan Sulfate

Purification of proteoglycans. Proteoglycans (PG) in the extracellular matrix (ECM) of the two cell lines were labeled with 20 μCi/ml ^{3}H-glucosamine (to label the GAG backbone; DuPont NEN, Boston, MA) and 50 mCi/ml $Na_2^{35}SO_4$ (to label the sulfate groups; ICN Biomedicals Inc., Irvine, CA). PG in the ECM of M2-10B4 and AFT024 cells were released by treatment of the cell layers with 0.25% trypsin + 1 mM EDTA, followed by removal of the cells by centrifugation. PG were purified by anion exchange HPLC as previously described (24, 25).

Preparation of heparan sulfate. Purified PG were digested with chondroitinase ABC (cABC; Seikagaku America Inc.), which digests chondroitin and dermatan sulfate, and chromatographed on a

Sephadex G-50 column. To cleave heparan sulfate side chains from the core protein, the material eluting at V_0 was treated with sodium hydroxide in the presence of sodium borohydride ($NaBH_4$), and free heparan sulfate (HS) chains released from the core protein were obtained by rechromatography on a Sephadex G-50 column (24, 25).

Estimation of size of HS. The size of HS was estimated by gel filtration chromatography on a Sepharose CL-6B column equilibrated in 4 M guanidine hydrochloride and 0.05 M sodium acetate, pH 5.8, as described earlier (24, 25). The approximate size of HS was estimated by the method of Wasteson (28).

Distribution of N-sulfation and O-sulfation in HS. HS from both cell lines was subjected to low pH nitrous acid (pH 1.5) deaminative cleavage of N-sulfated regions as described (29). The digested oligosaccharides were resolved by gel filtration chromatography on a Sephadex G-25 column equilibrated in 0.2 M ammonium acetate, pH 7.0. The resulting oligosaccharides were analyzed as described in detail earlier (25, 30).

Statistics

Results of experimental points from different experiments are reported as the mean ± standard error of the mean (SEM). Significance levels were determined by either paired or nonpaired Student's *t*-test analysis as indicated.

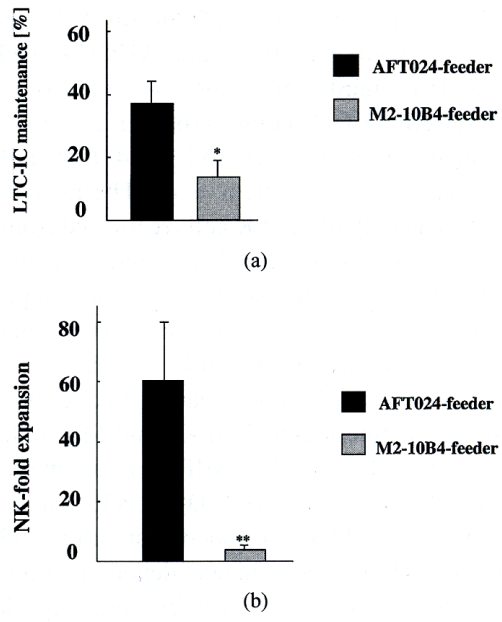

Figure 1. Primitive human myeloid (LTC-IC) and lymphoid (NK-IC) progenitors are maintained better when cultured in contact with the murine fetal liver cell line AFT024 compared to M2-10B4-marrow feeders. (a) 10,000 CD34+/HLA-DR-cells were cultured in contact with AFT024 or M2-10B4 feeders (n = 6). After five weeks cells were harvested and replated in limiting dilutions onto M2-10B4 feeders to assess the frequency of LTC-IC. Maintenance of LTC-IC was determined by comparing the absolute number of LTC-IC on day 0 with the absolute number of LTC-IC at week 5; (b) 10,000 CD34+/HLA-DR−/CD2−/CD7− cells were plated in contact with AFT024 (n = 6) or M2-10B4 feeders (n = 4). Cultures were maintained in lymphoid differentiation medium. After five weeks, adherent and nonadherent cells were harvested and stained with anti-CD56-PE and anti-CD3-FITC antibodies and analyzed by Flowcytometry to determine the presence of CD56+/CD3− NK cells. Cells were also examined for their capability to kill K562 targets. NK-fold expansion was calculated as the number of mature NK-cells present after five weeks divided by the number of these cells at day 0. Statistical analysis: Results are shown as mean ± SEM. Comparison made between M2-10B4 cultures and AFT024 cultures ($^*p < 0.05$, $^{**}p < 0.01$).

RESULTS

The Murine Fetal Liver Cell Line, AFT024, Supports Primitive Myeloid and Lymphoid Progenitors Better than M2-10B4

In initial experiments, we compared the ability of the cell lines AFT024 and M2-10B4 to support maintenance of primitive myeloid as well as lymphoid progenitors from adult human bone marrow in stroma contact cultures (Figure 1a). CD34+/HLA-DR− cells were cultured for five weeks on AFT024 or M2-10B4 feeders without any cytokines and assessed for maintenance of primitive myeloid Long-term Culture Initiating Cells (LTC-IC). We found 17.5 ± 2.0% of input LTC-IC were maintained in M2-10B4 contact cultures compared to 39.7 ± 6.2% LTC-IC maintenance in AFT024 contact cultures ($p = 0.01$, n = 6). We also cultured lymphoid depleted CD34+/HLA-DR−/CD2−/CD7− cells in

lymphoid differentiation medium for 5–6 weeks in contact with M2-10B4 and AFT024 feeders (Figure 1b). Significantly more NK-cells were recovered from AFT024 cultures (60.6 ± 21.1 fold expansion, n = 6) compared to M2-10B4 (6.95 ± 0.40 fold expansion, $p < 0.01$, n = 4). The phenotype and lytic capacity of the NK cells recovered from either culture was equivalent. These studies show that growth of primitive human myeloid and lymphoid progenitors is supported better in AFT024 contact cultures than in M2-10B4 contact cultures.

We have previously shown that a cytokine cocktail consistent of Interleukin-3 (IL-3) and Macrophage Inflammatory Protein 1α (MIP-1α) can maintain all LTC-IC in CD34+/HLA-DR− cells for up to eight weeks in M2-10B4 noncontact cultures (31). To assess LTC-IC maintenance, depending only on progenitor adhesion and proliferation, we added these cytokines to both glutaraldehyde-fixed and metabolically inactive as well as viable AFT024 and M2-10B4 contact cultures. LTC-IC maintenance was significantly lower when CD34+/HLA-DR− cells were cultured on fixed AFT024 (40.0 ± 6.7; $p < 0.01$) or M2-10B4 feeders (56.0 ± 9.9; $p = 0.01$) in medium containing IL-3 and MIP-1α. In contrast, when cells were cultured in wells without adhesive matrix (Bovine serum albumine coated) 88.9 ± 12.4% of LTC-IC could be maintained (Figure 2). However, 149 ± 17% LTC-IC were recovered from viable AFT024 cultures, whereas only 35.7 ± 3.5% LTC-IC could be recovered after five weeks from viable M2-10B4 contact cultures (n = 6, $p < 0.005$).

Despite Similiar Adhesion of Human Progenitors to Both Feeders, AFT024 Stromal Factors Counteract Contact Mediated Inhibition of Proliferation

Next, we asked whether better LTC-IC maintenance in AFT024 contact cultures may be due to less adhesive interactions of primitive hematopoietic progenitors and therefore less integrin mediated growth inhibition (19, 20). To answer this question

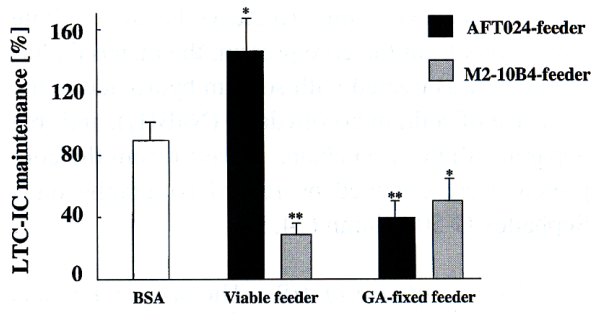

Figure 2. In contrast to cultures on fixed stroma and viable M2-10B4 contact cultures with viable AFT024 feeders not only maintain but also expand LTC-IC after five weeks. A total of 10,000 CD34+/HLA-DR− cells were plated in contact with viable as well as GA-fixed AFT024-feeders or viable as well as GA-fixed M2-10B4 feeders. Additionally 10,000 CD34+/HLA-DR− cells were plated in culture dishes precoated with BSA instead of stromal feeders. After five weeks, cells were harvested and replated in limiting dilutions onto M2-10B4 feeders to assess the frequency of LTC-IC. Maintenance of LTC-IC was determined by comparing the absolute number of LTC-IC on day 0 with the absolute number of LTC-IC at week 5 (n = 6). Statistical analysis: Results are shown as mean ± SEM. Comparison made between stromal feeders and BSA (*$p < 0.05$, **$p < 0.01$).

we assessed the adhesion of hematopoietic progenitors and cell cycle status in the adherent and nonadherent fraction of AFT024 and M2-10B4 feeders (19, 20). Bovine serum albumine (BSA) and poly-L-lysine coated plates were used as controls. As shown in Figure 3, CD34+ cells adhered to fixed (37.6 ± 3.4%) and viable AFT024 feeders (42.1 ± 3.5%) at

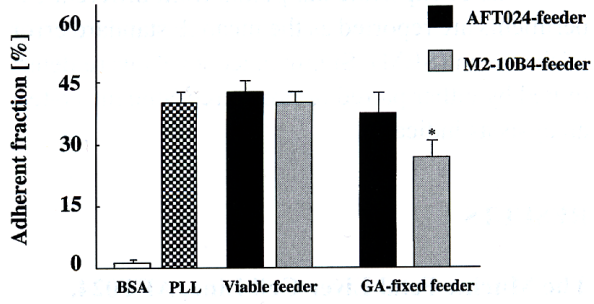

Figure 3. Adhesion of human progenitors to viable or fixed stromal feeders as well as to poly-L-lysin (PLL) is similar. A total of 200,000 CD34+ cells per point were plated in 24-well plates containing either viable or fixed confluent M2-10B4 or AFT024 feeders. After 12–16 hours the nonadherent fraction was removed and the adherent fraction was collected and counted in a hemocytometer. In control experiments adhesion to BSA or poly-L-lysine coated plates was measured. Comparisons made between viable and fixed feeders (*$p < 0.05$, **$p < 0.01$).

least as well as to fixed (25.1 ± 3.3%) and viable M2-10B4 feeders (41.1 ± 3.4%; n.s.), indicating that adhesive interactions may not explain the differences in LTC-IC maintenance.

Next, we determined the effect of adhesion to AFT024 and M2-10B4 feeders on progenitor proliferation by measuring the cell cycle status (cells in S+G2/M-phase) of CD34+ cells cultured for 12–16 hours with fixed and viable AFT024 and M2-10B4 feeders. Cell cycle progression of cells cocultured with fixed AFT024 feeders (14.6 ± 3.6%) or fixed M2-10B4 feeders (13.4 ± 3.1%) in the presence of IL-3 and MIP-1α was inhibited compared to BSA (24.1 ± 3.0%; $p < 0.05$) and PLL cultures (19.9 ± 3.0%; $p < 0.05$). In addition, the number of cycling cells present in the adherent fraction on M2-10B4-feeders (21.2 ± 2.3%) was also significantly lower as compared to BSA. In addition, cells adherent to viable AFT024 feeders were even stimulated to enter cell cycle (29.8 ± 1.7%, $p < 0.05$ compared to BSA).

As shown in Figure 4, the cell cycle entrance in the nonadherent fraction was not inhibited in supernatants of fixed (30.8 ± 4.2% for AFT024 and 28.8 ± 3.2% for M210-B4, n.s.) or viable cell lines (34.8 ± 3.9% for AFT024 and 35.6 ± 4.9% for M210-B4, n.s.) when compared to BSA (27.5 ± 3.6%; n.s.). This confirms our previous reports that cells in supernatants of several cell lines are maintained better in noncontact conditions due to increased cell cycle progression (5, 19–21, 23, 32). The stem cell supporting microenvironment of AFT024 however overrides contact mediated inhibition of proliferation (29.8 ± 1.7% cell cycle entrance in contact with viable AFT024 compared to 21.1 ± 2.3% in contact with M210-B4; $p < 0.05$).

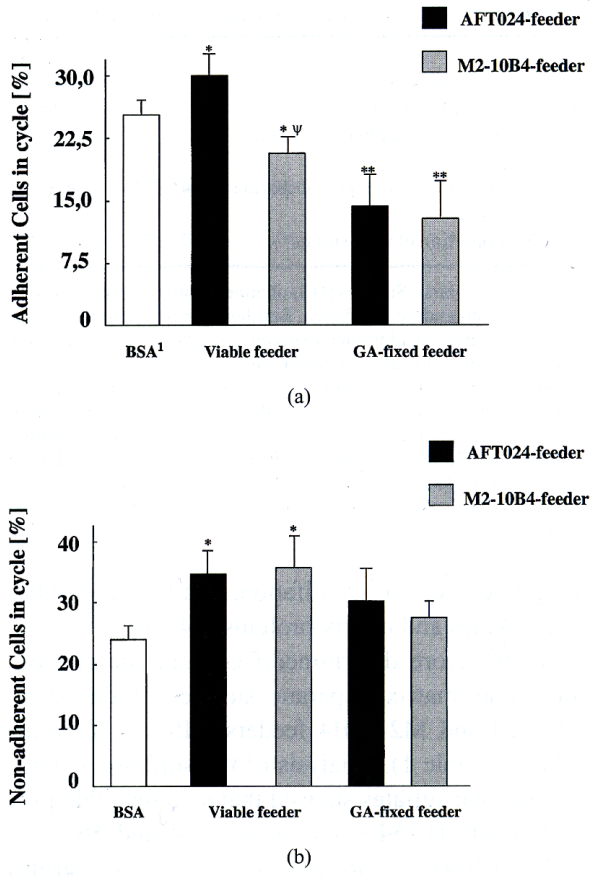

Figure 4. Adherent and nonadherent cells were harvested separately, labeled with anti CD34-FITC, permeabilized and stained with propidiumiodide. The fraction of CD34+ cells in G0/G1-, S- and G2/M phase was assessed by flow cytometry. In contrast to cells cocultured with M2-10B4 feeders there is no inhibition of cell cycle progression for CD34+ cells adherent to viable AFT024 feeders when compared to the nonadherent fraction. In addition, significantly more CD34+ cells adherent to viable AFT024-feeders are in cell cycle compared to cultures with BSA. ($^*p < 0.05$, $^{**}p < 0.01$ compared to BSA, $^*<0.05$ compared to the nonadherent fraction). In Figures 4a and b all cells cultured in wells that have been coated with BSA did not adhere and belong to the nonadherent fraction.

Large 6-O-Sulfated Heparan Sulfates Could in Part be Responsible for the Increased LTC-IC Maintenance in Contact Cultures with Viable AFT024

We next asked which factors are responsible for the increased proliferation in nonfixed AFT024 contact cultures. We and others have previously shown a similar production of several murine cytokines, such as IL-1β, IL-3, IL-6, IL-10, IL-12p40, IL-12p70, GM-CSF, MIP-1α, MIP-1β, MIP-2, TNFα, TPO, Flt-3L, and SCF in supportive and less supportive feeders (4, 15). In addition, we also demonstrated that stromal heparance sulfates that are present in M2-10B4 supernatants are large, have

TABLE 1 Analysis of Heparan Sulfates in Stromal Feeders

	AFT024	M2-10B4
Average size of HS [kDa]	34	17
Analysis of O-sulfated oligosaccharides of HS		
3-H (proportion of total poysaccharide backbone*)	54 ± 3%	56 ±2%
35-S (proportion of total sulfate*)	27 ± 2%	27 ± 2%

Analysis of Heparan Sulfates (HS) obtained from one representative purification procedure are shown. Similar figures were obtained for size and sulfation in additional experiments. The average size of HS is that of the major peak in each sample.
*Proteoglycans in these samples eluted as 2–3 separate peaks on anion exchange HPLC purification. HS was purified separately from each peak, digested using nitrous acid and analyzed for O-sulfation pattern by gel filtration chromatography. The pattern of O-sulfation was similar in HS in the various HPLC peaks from each sample.
Data are shown as the mean ± SEM of the analysis of the individual peaks.

a high degree of 6-O-sulfation, and bind a number of cytokines and matrix proteins (24–26).

We therefore determinedif cell surface and extracellular matrix heparane sulfates produced by AFT024 and M2-10B4 feeders differ in these attributes (Table 1). Analysis of the sulfation pattern of heparane sulfates showed that a comparable proportion of ^{3}H (54 ± 3% in AFT024 and 56 ± 3% in M2-10B4) are present in the oligosaccharides 3–8 monosaccharides) of both cell lines. This indicates that a large but comparable proportion of the polysaccharide backbone structures from both cell lines is comprised of such regions. The proportion of ^{35}S present in these oligosaccharides was also comparable for AFT024 and M2-10B4 (27 ± 2% in both), indicating that the heparane sulfates from both cell lines have an equivalent extent of O-sulfation, largely 6-O-sulfate. Thus, the extent of heparane sulfate O-sulfation in AFT024 matrix is similar to that present in M2-10B4 supernatant. However, the size of HS (34 kDa) in the cell as well as in the extracellular matrix extract of AFT024 cells was two-fold larger than that of M2-10B4 HS (17 kDa).

DISCUSSION

In this study we demonstrate for the first time that maintenance and expansion of primitive human progenitors supported by stromal feeders can be related to proliferation and cell cycle progression while adherent to the extracellular matrix and stromal cells. Primitive human bone marrow cells that have been cultured for five weeks in contact with AFT024 feeders are maintained significantly better compared to cultures in contact with the murine marrow feeder M2-10B4. This is due to factors expressed on viable AFT024-cells that counteract contact mediated inhibition of proliferation.

The superior support of the AFT024-feeder layer especially for the primitive hematopoiesis has been shown before by Moore et al. in the murine system (13). In humans, Thiemann et al. observed a 2–6 fold higher frequency of Extended-Long-term Culture Initiating Cells after a 3–4 week culture period in AFT024 contact cultures compared to primary stroma (14). We described a significantly higher readout of Long-term Culture Initiating cells after long-term cultures on AFT024-feeders compared to M2-10B4 (17). We also demonstrated that AFT024 feeders allow generation of multiple secondary regenerative multilineage progenitors derived from one single sorted human CD34+/HLA-DR−/Lin− bone marrow cell (16). However the mechanism behind this support are unknown.

A number of studies have shown that maintenance of primitive human hematopoiesis is lower when cultured in contact with the stroma compared to noncontact conditions (4, 19, 21). We demonstrated that maintenance of these primitive progenitors in noncontact conditions is due to extensive proliferation of a small subpopulation of conserved progenitors while the majority of these cells were lost (32). We have attributed the decrease in progenitor maintenance in contact cultures to contact mediated inhibition of progenitor growth. This decrease in proliferation is due to engagement of β1-integrins on CD34+ cells with components of the extracellular matrix, such as fibronectin, that leads to an upregulation of $p27^{Kip1}$ blocking progression through the R point of the S-phase of the cell cycle (22). Thus, decreased adhesion to AFT024 feeders and therefore less engagement of β1-integrins on CD34+ cells could have been one reason for the increased

maintenance of primitive progenitors in AFT024 contact cultures. However, we demonstrate in this study that the fraction of cells which is adherent to AFT024 and M2-10B4 feeders, is similar. We further demonstrate that contact between hematopoietic cells and adhesive ligands in fixed AFT024 feeders causes a similar inhibition of cell cycle progression as what we observed on M2-10B4 feeders. Thus, adhesion to fixed AFT024 cells and extracellular matrix components as well as to M2-10B4 does occur and this adhesion causes similar inhibition of proliferation subsequently followed by decreased maintenance of primitive Long-term Culture Initiating Cells.

These results confirm previous experiments that demonstrated cell cycle inhibition in primitive human hematopoietic cells due to adhesion to purified fibronectin components (20–23). However, significantly more cells that adhere to viable AFT024 cells progressed through cell cycle compared to those that adhere to glutaraldehyde fixed feeders. Thus, continued proliferation of adherent cells cultured on viable AFT024 feeders may explain the improved Long-term Culture Initiating Cell maintenance. Further, the addition of IL-3 and MIP-1α to viable AFT024 cultures resulted in an increased proliferation in contact cultures resulting in better maintenance of Long-term Culture Initiating Cells that even exceeded that seen in wells without adhesive interactions. Thus, one or more factors present in contact with viable AFT024 cells not only counteracted contact mediated inhibition of primitive progenitor growth but further improved support for these cells.

We recently demonstrated that human hematopoietic cells by themselves are able to induce the release of additional factors in stromal supernatants that even extends the hematopoietic support of these feeders (5, 15, 33). Here we demonstrate that such factors are able to override contact mediated proliferation inhibition resulting in expansion of human marrow derived Long-term Culture Initiating Cells.

The nature of these factors are unkown so far. Using a subtractive hybridization approach Moore et al. identified cDNAs in AFT024 but not in nonsupportive cell lines that encode for novel growth factors and extracellular matrix proteins which may be responsible for the effect seen in our cultures (34–36). One of the factors is the murine delta-like-1 (mdlk1) protein which increases the *ex vivo* support of primitive murine hematopoietic progenitors when ectopically expressed in a hematopoietic nonsupportive cell line (34). Han et al. have shown that ligation of the human Delta ligand (hDll1) with its receptor Notch increased the progression into cell cycle of primitive (Lin-) murine hematopoietic progenitors. This resulted in a 3-fold expansion of Colony forming cells *in vitro* (HP-CFC) and in the spleen of the mice (CFU-S). In addition, less apoptosis and differentiation was seen when compared to the control experiments (37).

But additional factors present in AFT024 feeders may also be responsible for the better supportive capacity of this feeder (35, 36). We have previously shown that supernatants supportive for primitive human hematopoiesis contain large (>30 kDa) 6-O-sulfated heparane sulfate glycosaminoglycans whereas nonsupportive supernatants contain only small heparane sulfate glycosaminoglycans (25). Here we show that similar large size 6-O-sulfated heparane sulfate glycosaminoglycans (34 kDa) are present in the extracellular matrix of AFT024 but not in M2-10B4. Using an affinity coelectrophoresis assay, we have shown that these large size sugars bind IL-3 and MIP-1α with much greater avidity compared to small size molecules (25). Consistent with our studies, Drzeniek et al. identified three populations of heparan sulfate proteoglycans larger than 30 kDa in the cell extract of MS-5 stromal cells, that also supports very primitive human hematopoiesis in contact cultures (3, 38). Some of the novel factors identified by Moore et al. that are present only in AFT024 have been identified as proteoglycans as well (35, 36). Thus, we speculate that AFT024 specific large 6-O-sulfated heparan sulfate proteoglycans may sequester and present growth factors required for proliferation of immature progenitors that are adherent to the stromal feeder. In addition, factors secreted by human hematopoietic cells and/or some of the cytokines added to the medium may modulate proteoglycan synthesis and GAG structures in

the culture system: Cytokines differentially regulate gene expression of proteoglycan core proteins (39–41), as well as alter size (39) and sulfation (42) of the side chains of these proteins. This can result in significant changes in the protein binding, adhesive and growth regulatory functions of these proteoglycans. A number of such cytokines and chemokines may be produced by the primitive human hematopoietic cells themselves (43).

Thus, we hypothesize that in contrast to M2-10B4-feeders the synergistic interactions between the primitive progenitors and AFT024-proteoglycans could be responsible for the superior maintenance of primitive human hematopoiesis when cultured on confluent AFT024-feeders. Futher identification of factors present in AFT024 will give insights in the regulation of hematopoietic stem cells and may help optimize culture conditions for *in vitro* stem cell expansion and retroviral gene transduction.

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Functional analysis of initial cell divisions defines the subsequent fate of individual human CD34$^+$CD38$^-$ cells

Michael Punzel,* Tao Zhang,* Daihong Liu, Volker Eckstein, and Anthony D. Ho

Department of Medicine V, University of Heidelberg, Heidelberg, Germany

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Objective. **We assessed the relationship of individual cell divisional behavior with the functional fate of stem cell candidates at the single cell level.**

Materials and Methods. **Individual CD34$^+$CD38$^-$ cells derived from cord blood (88–352 cells in each of 25 experiments) were cultured in early-acting conditioned medium (EACM) or late-acting proliferation medium (LAPM). The initial cell divisions were microscopically monitored every 12 to 24 hours and then assessed for primitive function in the myeloid lymphoid-initiating cell assay and committed function in the colony-forming cell (CFC) assay.**

Results. **Despite a higher proliferative capacity in LAPM, significantly more quiescent cells (11.1 ± 1.7%) were found in LAPM than in EACM cultures (1.1 ± 0.4%; $p < 0.001$). No differences were observed in the initially plated CD34$^+$Cd38$^-$ cells that produced asymmetrically dividing progeny. The majority of cells with subsequent ML-IC function divided in EACM but were found among quiescent cells in LAPM conditions. All cycling cells with subsequent ML-IC capacity initially remained quiescent for at least 96 hours. All ML-IC had been recruited exclusively (100%) from either cytokine nonresponsive (quiescent) or slow and asymmetrically dividing cells (1–2 divisions). In contrast, the majority of CFCs entered the cell cycle immediately after plating, have divided more than two times, and only 20.2 ± 5.5% of the cycled CFC divided asymmetrically.**

Conclusions. **Asymmetric divisional behavior of CD34$^+$CD38$^-$cells cannot be influenced by culture conditions. Primitive ML-IC can be distinguished from committed CFC by initial quiescence or asymmetric divisions. Committed CFC cycle rapidly and symmetrically.** **© 2002 International Society for Experimental Hematology. Published by Elsevier Science Inc.**

Hematopoietic stem cells (HSC) are characterized by the ability to self-renew and differentiate into all hematopoietic lineages. The most primitive stem cells probably are quiescent or have very slow cycling rates [1–4] but possess the ability to enter into the cell cycle and efficiently produce progeny cells upon exposure to appropriate signals [5–7]. In murine models, these cells have been identified by serial competitive repopulation assays [8–13]. Because serial transplants are not possible in humans, several in vivo and in vitro assays have been developed to provide surrogate tools to study human hematopoiesis [14–20]. Such assays only allow an approximate estimate of the HSC frequency. As all HSC assays are retrospective, the initial identification of human HSC is not yet possible.

To overcome these limitations, an increasing effort has been made to correlate prospectively the initial divisional behavior of purified hematopoietic progenitor cells in vitro with their functional properties. Flow cytometric analysis of membrane dye resolution has been used to monitor the divisional history of purified hematopoietic progenitors and to subsequently isolate cells with distinct divisional characteristics. It has been demonstrated that after several divisions, HSC loses its primitive function in vitro and its engraftment ability in NOD/SCID mice [2,21–26]. However, interpretation of cell divisional history according to membrane dye resolution of PKH-26 or carboxyfluorescein diacetate succinimidyl ester (CFSE) in bulk cultures has been controversial [27].

Results obtained from studies using HSC from adult sources such as mobilized peripheral blood or bone marrow should be discussed in context of recent data demonstrating that more HSC from earlier stages in development can be found in the cell cycle [28,29]. Furthermore, bulk culture

Offprint requests to: Anthony D. Ho, M.D., Ph.D., Department of Medicine V, University of Heidelberg, Hospital Str. 3, Heidelberg, Germany 69115; E-mail: anthony_dick.ho@urz.uni-heidelberg.de

*The first two authors contributed equally to this work.

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approaches can only assess groups of cells that had undergone certain numbers of cell cycle transits. Neither the exact follow-up of divisional steps nor the onset of cell cycle transit can be defined using this method. Thus, to draw definite conclusions, the exact divisional history of each daughter cell generation, including asymmetric division pattern, must be determined.

Some studies have investigated functional properties in the context of individual cell divisional behavior. Phenotypic analysis of human cord blood stem cell candidate cells demonstrated self-renewing versus differentiating divisions with respect to the stem cell phenotype of first-generation daughter cells in response to late-acting and early-acting cytokines [30]. In single cell experiments investigating the proliferative potential of human fetal liver cells, cell cycle properties and replating potential were unevenly distributed among each generation of daughter cells, suggesting asymmetric distribution of functional properties [31,32]. Similarly, we and others demonstrated that asymmetric divisions occur in hematopoietic progenitors and seemed to be related to more primitive function [32–37]. However, no direct correlation between cell cycle status, asymmetric divisions, and distinct functional properties at the single cell level has been shown.

In this study, we demonstrate a novel in vitro system that allows direct visual monitoring of the initial divisional behavior of single human hematopoietic cells followed by subsequent functional assessment of each individual cultured cell. In contrast to bulk experiments [24,25,28,38,39], our system uses microscopic documentation of divisional history combined with a direct functional readout including frequency assessment of different progenitor types. To assess the most primitive in vitro function for human hematopoietic cells available, we applied our recently developed myeloid lymphoid-initiating cell (ML-IC) assay [40]. This assay determines the frequency of individual self-renewing cells capable of generating multiple (at least two) secondary myeloid long-term culture-initiating cells (LTC-IC) as well as multiple secondary "lymphopoiesis-initiating cells" [41] in a given population and, therefore, is closely related to human HSC. In contrast, committed and lineage-determined function was measured by means of colony-forming cells (CFC).

Culturing individually sorted $CD34^+CD38^-$ cells in early-acting versus late-acting cytokine conditions, we demonstrated in this study that ML-IC and CFC capacities are unequivocally related to individual cells with distinct divisional history.

Materials and methods

Cell source and preparation

Human cord blood was collected from the umbilical cord after obtaining informed consent using guidelines approved by the Ethics Committee on the Use of Human Subjects at the University of Heidelberg. Mononuclear cells were obtained by Ficoll-Hypaque (Sigma-Diagnostics, St. Louis, MO, USA) centrifugation. $CD34^+$ cells were selected with a monoclonal anti-CD34 antibody labeled with magnetic beads and separated by a magnetic column (Miltenyi-Biotech, Bergisch-Gladbach, Germany).

Single cell deposition cultures

Individual $CD34^+CD38^-PKH^{bright}$ cells were sorted into bovine serum albumin-coated 96-well plates (Nunc) using the automatic cell deposition unit (ACDU) on a FACS Vantage flow cytometry system (Becton-Dickinson). Cells were cultured in either early-acting conditioned medium (EACM) or late-acting proliferation medium (LAPM). The $CD34^+CD38^-$ cells were stained with PKH-26 only to assist monitoring of asymmetric divisional behavior if visual analysis and monitoring failed to reveal whether the division was symmetric or asymmetric. The divisional history of each initially deposited cell was monitored visually during the first 10 days of culture by microscopic tracking every 12 to 24 hours and subsequently transferred in either ML-IC or CFC-readouts. Because this experimental setup allows conclusions only on the initially plated "mother cell," the entire progeny of each individually plated cell was used for functional analysis. Thus, assessment of ML-IC and CFC was done in separate experiments.

EACM. This medium was previously developed by our group to replace stroma-conditioned medium by defined culture conditions [42–44]. We have identified cytokines and extracellular matrix components in medium that was conditioned by human bone marrow stroma or M2-10B4 feeders. Based on this information, we generated our so-called "reconstituted stroma conditioned medium" [42]: long-term bone marrow culture (LTBMC) medium was supplemented with human cytokines at concentrations found in supernatants of human bone marrow stroma: 10 pg/mL granulocyte-macrophage colony-stimulating factor (Immunex Corp., Seattle, WA, USA), 250 pg/mL granulocyte colony-stimulating factor (Amgen, Thousand Oaks, CA, USA), 200 pg/mL stem cell factor (R&D Systems, Minneapolis, MN, USA), 50 pg/mL leukemia inhibitory factor (LIF) (R&D Systems), 200 pg/mL macrophage inflammatory protein-1α (R&D Systems), and 50 pg/mL interleukin-6 (Cell Systems, St. Katharinen, Germany); as well as O-sulfated glycosaminoglycans [10 μg/ml N-desulfated, N-acetylated (NDSNAc) heparin (Seikagaku America Inc., Rockville, MD, USA)]. We added 25 ng/mL Flt3L (a kind gift from Immunex) and 25 ng/mL thrombopoietin (Cell Systems) to the medium and termed it early-acting conditioned medium (EACM).

LAPM. This medium was used as described in our previous work assessing asymmetric divisions of human hematopoietic cells [32]. We used the myeloid long-term culture medium Myelocult (Stem Cell Technology, Vancouver, BC, Canada) supplemented with 2.5 U/mL erythropoietin (Amgen), 10 ng/mL interleukin-3 (Cell Systems), 500 U/mL interleukin-6, 10 ng/mL granulocyte-macrophage colony-stimulating factor (Cell Systems), 2.5 ng/mL basic fibroblast growth factor (Cell Systems), 10 ng/mL insulin-like growth factor I (Cell Systems), 50 ng/mL stem cell factor, 1000 U/mL penicillin, and 100 U/mL streptomycin (Gibco) [32].

ML-IC assay

After observing the cultures in EACM or LAPM for 5 to 10 days, cells were transferred to the ML-IC assay (Fig. 1), as described previously [40]. Briefly, cells were transferred from EACM or LAPM cultures in secondary plates containing AFT024 feeders (a kind gift from I. Lemischka, described in [40]). After another 2 weeks of proliferation, the content of each single well of the sec-

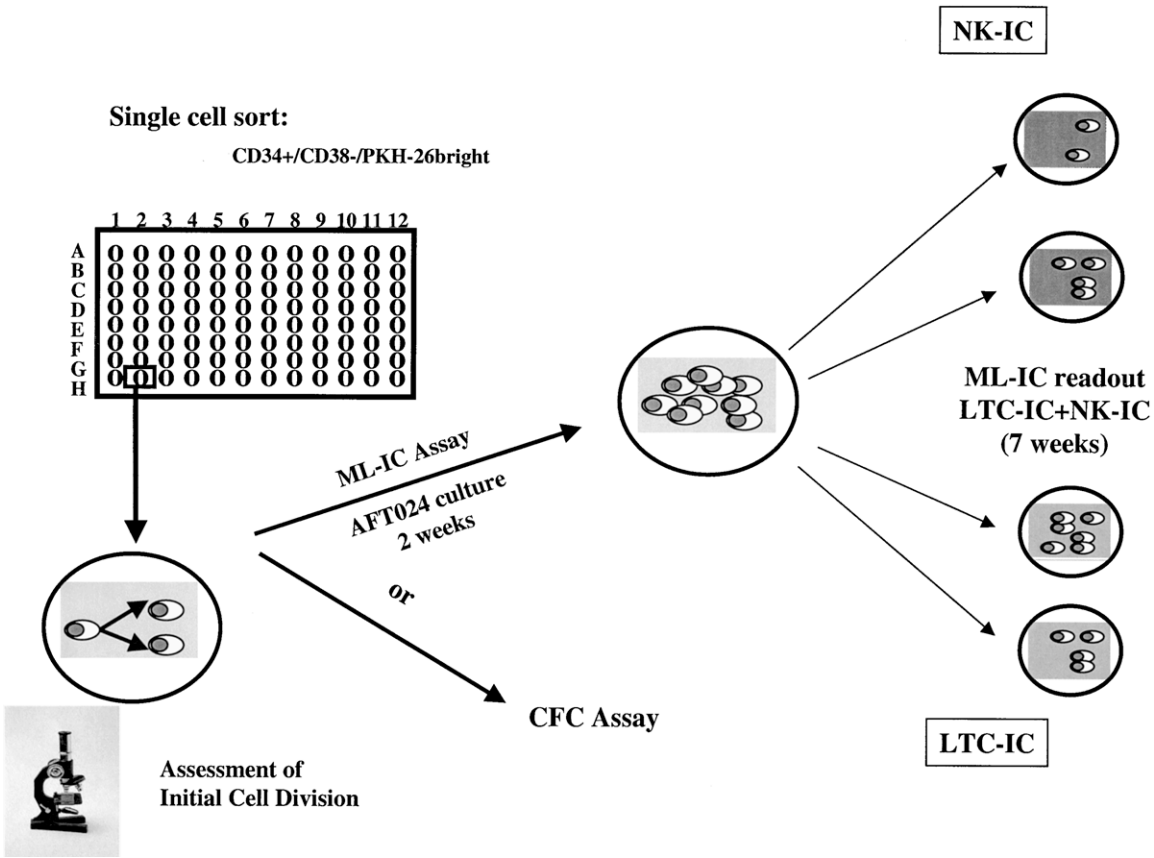

Figure 1. Experimental setup. $CD34^+CD38^-$ cells were individually sorted onto 96-well plates (one cell per well) in either EACM or LAPM conditions. Divisional history within 5 to 10 days of culture was determined by dye resolution fluorescence microscopy. After the divisional history for each cell was documented, all progeny of initially sorted cells were transferred to the same location of secondary 96-well plates prelayered with the murine fetal liver feeder AFT024 to assess myeloid lymphoid-initiating cells (ML-IC). After 2 weeks of AFT024 culture, the content of each well was divided into four equal parts by volume and equally distributed to the same location of four tertiary 96-well plates to assess LTC-IC and NK-IC in a tertiary 7-week culture step. If wells tested positive for LTC-IC and NK-IC derived from the same initially sorted cell, the individual progenitor was named as one ML-IC. To assess CFC frequency, wells of the primary 96-well plates were overlaid with clonogeneic methylcellulose medium and scored for colony formation after another 12 to 14 days. In other experiments, ML-IC were detected up front from the starting population by sorting $CD34^+CD38^-$ cells directly onto 96-well plates prelayered with the murine fetal liver feeder AFT024.

ondary plates was harvested with trypsin and divided equally over four tertiary 96-well plates containing irradiated AFT024 feeders such that that one fourth of each single cell progeny was deposited in the identical location in the four tertiary plates. The four tertiary plates had been established with a confluent layer of AFT024 cells, two with lymphoid differentiation medium for natural killer initiating cell (NK-IC) readout [40,41,45] and the other two with myeloid differentiation medium for LTC-IC readout [40]. Detection of at least one LTC-IC and one NK-IC in the identical location in the tertiary plates was required to consider the initially plated cord blood $CD34^+CD38^-$ cell as ML-IC [40].

Definition of cell divisional history

We analyzed asymmetric divisions based on the microscopic observation of individual cell proliferation history as well as membrane dye distribution of the next two daughter cell generations. We defined a cell division as asymmetric if one of the first daughter cells did not divide or died while the other daughter cell divided subsequently after an observation period up to 10 days. We defined a division as asynchronous but symmetric if there was only a time delay between the division onset of the two daughter cells with symmetric distribution of the membrane dye between each of the four second-generation daughter cells.

Statistical analysis

Results of experimental points from different experiments are expressed as mean ± SEM. Significance levels were determined by either paired or nonpaired two-sided Student's *t*-test analysis as indicated.

Results

Individual $CD34^+CD38^-$ cells divide differently when exposed to early-acting versus late-acting cytokines

Individual $CD34^+CD38^-/PKH^{bright}$ cells derived from human cord blood were deposited into 96-well plates. Each experiment was performed using the same individual cord blood sample. In some cases, up to three individual cords

were pooled due to the low cell count and considered as one experiment. In each experiment (n = 24–29), one single cell was deposited onto 88 to 352 wells of 96-well plates per single experiment. The number of wells that contained one cell after sorting (29–85% of "positive" wells per experiment) are referred to as the actual number of cells deposited (50–221 cells per single experiment). Cells then were monitored for individual divisional behavior for up to 10 days.

We analyzed the clonal proliferation of each individual cultured cell as summarized in Table 1. In LAPM cultures, significantly more individually sorted cells had undergone more than two divisions during the observation period (fast dividing fraction), resulting in a higher fast dividing fraction (58.6 ± 4.4%) compared to EACM conditions (44.1 ± 4.6%; $p < 0.05$). In addition, we detected significantly more quiescent cells (11.1 ± 1.7%) in LAPM conditions compared to those cultured in EACM conditions (1.1 ± 0.4%; $p < 0.001$), indicating that LAPM conditions did not act on all of the sorted $CD34^+CD38^-$ cells. However, those cells that were responsive to cytokines revealed a higher cell cycle turnover compared to EACM conditions.

More cells in EACM conditions than LAPM cultures died during the observation period. This indicated that a fraction of cells were induced to undergo apoptosis in early-acting cytokine conditions only, not in LAPM conditions, suggesting that cytokine stimulation acts differentially on distinct subpopulations of the $CD34^+CD38^-$ fraction.

To analyze if the emerging differentiated cells change proliferative behavior when the individual cells are in contact with each other in bulk cultures, we also compared the overall expansion of 1000 $CD34^+CD38^-/PKH^{bright}$ cells in one culture well (n = 5). Similar to the single cell cultures, a significantly higher overall expansion (604- ± 207-fold) could be found in LAPM conditions compared to EACM cultures (170- ± 147-fold; $p < 0.05$). In FACS analysis, more PKH^{bright} cells could be detected in LAPM conditions compared to EACM conditions, suggesting that similar to the single cell experiments (Table 1), significant numbers of initially sorted cells remained quiescent in bulk cultures.

Table 1. Cell division of $CD34^+/CD38^-$ cells at single-cell level in late-acting proliferation medium (LAPM) and early-acting conditioned medium (EACM)‡

Day	Medium	n	Quiescent	SDF (≤2 divisions)	FDF (>2 divisions)
5	EACM	29	14.9 ± 2.3%†	67.9 ± 1.9%†	10.7 ± 2.2%†
	LAPM	28	33.9 ± 3.3%	38.0 ± 1.8%	23.9 ± 3.6%
10	EACM	25	1.1 ± 0.4%†	20.5 ± 1.8%	44.1 ± 4.6%*
	LAPM	24	11.1 ± 1.7%	20.0 ± 2.0%	58.6 ± 4.4%

Values are mean of relative numbers (%) per cells deposited ± SEM.
*$p < 0.05$ and †$p < 0.01$ compared to LAPM.
‡Cells that died during the observation period are not included in this table.
FDF = fast dividing fraction (defined as cells with >2 divisions); n = number of experiments (50–221 single cells per experiment); SDF = slow dividing fraction (defined as cells with ≤ divisions).

Asymmetric cell division is not influenced by cytokine combinations

We previously showed that asymmetric cell division is governed by intrinsic factors and did not change under the influence of several cytokines in vitro [32]. In this study, we compared the divisional behavior of $CD34^+CD38^-$ cells cultured with early-acting cytokines in EACM with conditions that contained late-acting cytokines (LAPM).

The results are summarized in Table 2, which indicates the cells showing asymmetric divisional behavior after 5 and 10 days of exposure to EACM (n = 25) or LAPM conditions (n = 24). Cell division was determined as asymmetric as defined in the Materials and methods section. In each of the experiments, 50 to 221 single cell divisions were analyzed. Despite the differences in proliferative potentials under different cytokine combinations, no differences in the number of asymmetrically dividing cells (8.4 ± 0.9% in LAPM compared to 10.0 ± 1.1% in EACM at day 5 and 20.0 ± 1.2% in LAPM compared to 21.3 ± 1.3% in EACM at day 10) were observed. In accordance with our previous observations, the present data confirmed that asymmetric cell division was not governed by extrinsic cytokine combinations.

Primitive ML-IC can be maintained independent of the initial divisional behavior

To determine the primitive and stem cell surrogate in vitro function of the initially cultured single $CD34^+CD38^-$ cells in relation to its divisional history, we transferred the entire progeny of each individual well into the ML-IC assay after documentation of cell division (Fig. 1). To assess ML-IC frequency in the freshly sorted population (day 0) independent of the cell division experiments, we sorted single $CD34^+CD38^-$ cells directly into the ML-IC assay. Thus, we were able to compare the frequency of ML-IC at day 0 with the frequency after the divisional culture at day 5. The results are summarized in Figure 2. Similar numbers of ML-IC could be detected in freshly sorted $CD34^+CD38^-$ cells (6.8 ± 1.0%) in cultures after either EACM (6.4 ± 1.6%; p = NS) or LAPM exposure (6.0 ± 1.9%; p = NS), indicating that primitive ML-IC are maintained in both culture conditions. However, by day 5, the divisional history of cells that subsequently could be determined as ML-IC was

Table 2. Asymmetric division of individual cultured CB $CD34^+/CD38^-$ cells

Day	Medium	n	Asymmetric division
5	EACM	29	10.0 ± 1.1%
	LAPM	28	8.4 ± 0.9%
10	EACM	25	21.3 ± 1.3%
	LAPM	24	20.0 ± 1.2%

Values are mean of relative numbers (%) of asymmetric dividing cells per cells deposited ± SEM.
n = number of experiments (50–221 single cells per experiment).

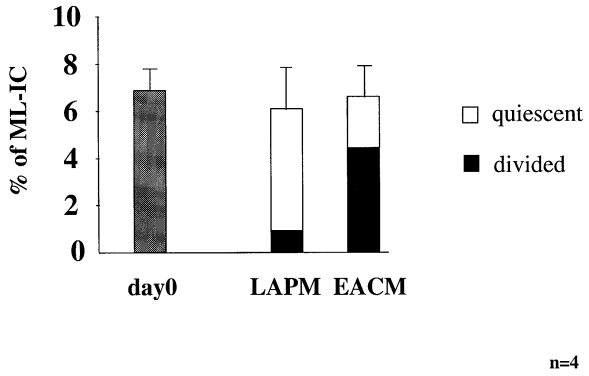

Figure 2. Primitive ML-IC can be stimulated to proliferate without loss of function. Individual $CD34^+CD38^-$ cells were sorted onto 96-well plates (one cell per well) in either EACM or LAPM conditions. Divisional history was monitored by dye resolution fluorescence microscopy. After 5 days, cells were transferred into the ML-IC assay, and primitive function was correlated with the divisional history for each single cell. To compare ML-IC frequency at days 0 and 5, cells also were cultured directly into the ML-IC-assay and ML-IC frequency assessed at day 0. After assessment of ML-IC in both culture conditions, the relationship between ML-IC derived from noncycling cells (quiescent ML-IC; white bars) and ML-IC derived from initially dividing cells (divided ML-IC; black bars) was determined.

significantly different. Most of the cells recognizable as ML-IC after culture in EACM conditions had divided (74.1 ± 9.6%; i.e., 23 of 34 ML-IC), whereas only 4 of the 31 ML-IC in LAPM conditions had divided (30.6 ± 23.3%, $p < 0.05$; n = 4).

ML-IC frequencies per total number of deposited cells in each subfraction for the 5-day culture period are summarized in Table 3. In both culture conditions, the frequency of ML-IC plummeted with every single round of cell cycle independent of the culture conditions. Even though most ML-IC in EACM conditions had divided, the highest ratio of ML-IC per deposited cell still was found in the quiescent population. Thus, the resolution capacity of this in vitro approach can precisely define the number of cycles that each individual cultured cell has undergone as well as the entire frequency of functional cells in a given population.

Table 3. Ratio of ML-IC/deposited cell in different proliferative subpopulations of $CD34^+/CD38^-$ cells

ML-IC	EACM (n = 4)	LAPM (n = 4)
Day 0 (% of total input)	6.8% ± 1.0	6.8% ± 1.0
Day 5 (% of total input)	6.4% ± 1.6	6.0% ± 1.9
ML-IC per quiescent cells (%)	14.0% ± 6.0	10.3% ± 3.5
ML-IC per cells with 1 division (%)	9.2% ± 2.2*	2.3% ± 1.0*
ML-IC per cells with 2 divisions (%)	1.6% ± 1.2†	0†

Values are mean of relative numbers (%) per cells deposited ± SEM.
*$p < 0.05$ and †$p < 0.01$ compared to quiescent ML-IC.

When cells were cultured over a 10-day period (n = 6), the total number of ML-IC plummeted in both culture conditions (1.62 ± 1.1% ML-IC in EACM; 2.70 ± 0.7% ML-IC in LAPM; p = NS). All ML-IC have cycled (≤3 times) in response to EACM conditions. In contrast, 40.3 ± 16.2% of all ML-IC remained quiescent over the entire 10-day culture period in LAPM. As shown in Figure 3 for LAPM conditions, those ML-IC that derived from cycling cells underwent only one or two rounds of cell divisions after a quiescent period of at least 96 hours. Five of six ML-IC assessed for divisional history demonstrated asymmetric divisions during the first two cell cycles. Similarly, 6 of 8 ML-IC that could be detected in EACM conditions divided asymmetrically. Thus, primitive ML-IC remained quiescent over 10 days in vitro or divided slowly and mainly asymmetrically with late onset of proliferation.

Primitive ML-IC and committed CFC are recruited from different subsets of progenitors with distinct divisional history

We determined the relationship between initial cell divisional behavior at a single cell level and the committed cell fate measured as CFC. These data were compared with those that yielded ML-IC. As shown in Figure 4, after 10 days of culture in LAPM, all ML-IC (100 ± 0%) could be detected in quiescent cells and in the slow dividing fraction (cells that divided fewer than three times) only. In contrast to ML-IC, only 7.3 ± 3.4% of cells that yielded CFC were initially determined in the quiescent/slow dividing fraction. Thus, 92.7 ± 3.4% of all CFC divided more than two times.

Taking all cell divisional properties together (Table 4), primitive ML-IC remain quiescent or have a late onset of proliferation that displays slow cell cycle throughput with mainly asymmetric divisions. In contrast, the majority of CFC (71.5 ± 10.4%) divided within the first 96 hours of culture and initially displayed mainly symmetric divisional behavior (20.2 ± 5.5% asymmetric divisions). This indicates that ML-IC and CFC are different not only in terms of initial cell cycle kinetics, but they also followed different intrinsic division programs.

Discussion

In this study, we correlated the immediate divisional history with long-term fate of primitive human hematopoietic progenitors at a single cell level. Several groups examined the relationship between divisional history and functional properties of progenitors out of bulk cultures using dye resolution tracking flow cytometry as indicators for cell division and cell cycle transit [23–25,28,39,46,47]. In contrast to these approaches, our novel experimental design applied membrane dye only to facilitate recognition of the divisional symmetry of individual cells. By direct monitoring, we followed the initial divisional behavior followed by subsequent analysis of different functional properties. Primitive

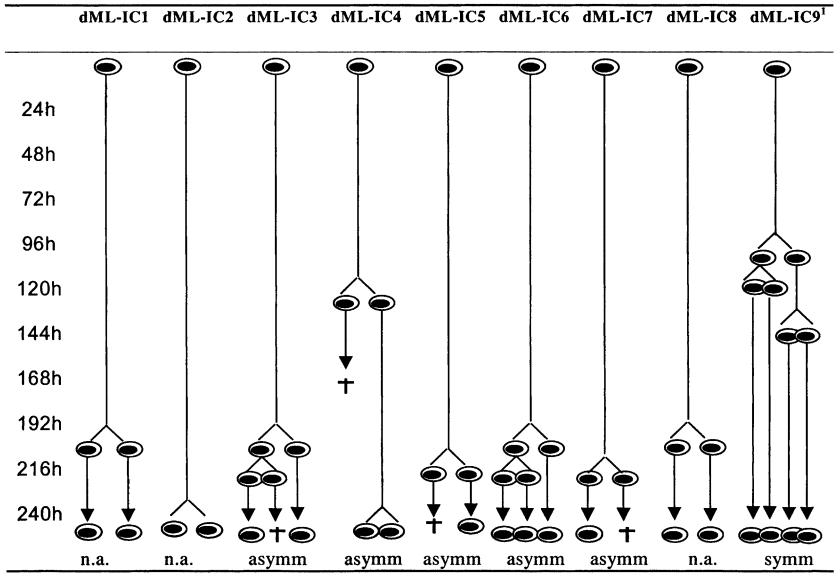

Figure 3. Divisional behavior of individual $CD34^{+}CD38^{-}$ cells with subsequent ML-IC function in LAPM with at least one division (dML-IC). Cells were sorted onto 96-well plates (one cell per well) in LAPM, and cell division was determined by microscopic observation every 12 to 24 hours. Dividing cells with subsequent ML-IC capacity (dML-IC; 9/569 individual cells in six independent experiments) remained initially quiescent for at least 96 hours and underwent fewer than three cycles over the 10-day observation period. In 5 of 6 cells in which division symmetry could be assessed, asymmetric divisions occurred. Time lapse microscopy revealed symmetric distribution of the membrane dye PKH-26 in an asynchronous but symmetric division with a time difference of less than 24 hours. †Cell death.

function was assessed using our recently developed ML-IC assay; committed function was assessed using the CFC assay. Thus, this experimental setup enabled us to quantitatively define the frequency of progenitors according to divisional history and asymmetric divisional behavior.

We unequivocally demonstrated that divisions of primitive human progenitor cells with ML-IC potential occurred without loss of function after 5 days of culture. In our LAPM conditions, 40% of all primitive progenitors subsequently yielding ML-IC remained quiescent over 10 days. All other ML-IC were identified among cells that divided slowly with one or two divisions and after at least 96 hours of initial quiescence. In agreement with our observation, Srour and others reported that based on dye resolution tracking cells that cycled extensively lost their engraftment potential as well as the ability to reinitiate long-term hematopoiesis in vitro [2,21–26]. Similar to our findings, Lanzkron et al. [48] reported that long-term repopulating cells remained quiescent for at least 48 hours after transplantation.

Using the dye carboxyfluorescein diacetate succinimidyl ester (CFSE) to track cell division, Glimm and Eaves [28] showed that the majority of human NOD/SCID repopulating stem cells, defined as competitive repopulating units, were identified among cells that underwent multiple, i.e., at least three, cell divisions after short-term culture. By transplanting fractions of cultured cells separated according to their proliferative history, they claimed that a substantial proportion of the total competitive repopulating units detectable in such cultured populations divided at least three times, suggesting that multiple self-renewing divisions in cultures were possible. However, controversies have arisen concerning the sensitivity of dye resolution as parameter for the threshold of rounds of cell divisions before the self-renewing capacity plummeted [26,28,39,49]. Most of these controversies are associated with the limitations of bulk cultures used to assess distinct cell fractions that underwent similar numbers of divisions. The fluorescence resolution of the dyes (CFSE or PKH) to discern differences between one single cell division, the heterogeneity of fluorescence among the cells with the same cycling history, and the patchy pattern of staining in each cell (unpublished observations) are inherent problems of defining cell divisions in bulk cultures. Other problems include the frequency estimation of cells with self-renewal capacity by limiting dilution and definition of an entire fraction of cells by fluorescence intensity itself without knowing the divisional behavior of the "mother cell" that gave rise to this fraction [27,49].

Targeting these limitations, we used the membrane dye PKH-26 only to facilitate recognition of individual cells after mitosis by fluorescence microscopy if the bright-field tracking of cell divisions did not visualize the divisional symmetry of two cell divisions. Monitoring the divisional history at the single cell level for 10 days with subsequent ML-IC and CFC assessment provided a direct functional correlation to the initially plated "mother cell."

Asymmetric divisions have been defined by different functional properties of the daughter cells [50–52]. Ema et al. [53] showed in the murine system that only one of two daughter cells gave rise to HSC activity, demonstrating the occurrence of self-renewing as well as asymmetric divisions

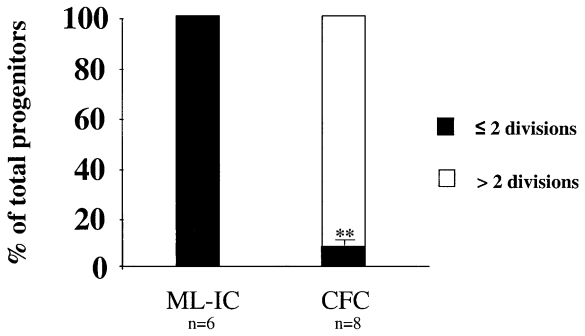

Figure 4. Primitive ML-IC and committed CFC have distinct proliferative histories. Individual $CD34^+CD38^-$ cells were sorted onto 96-well plates (one cell per well) in LAPM, and cell division was determined by dye resolution fluorescence microscopy. After 10 days of culture, the entire progeny of each individual well was transferred into the ML-IC assay (n = 6; 73–127 cells per experiment) to correlate primitive function with the divisional history for each single cell. In different plates, cells were overlaid with clonogeneic methylcellulose to assess the formation of CFC (n = 8; 56–208 cells per experiment). All primitive ML-IC (100%) were derived from single sorted progenitors that remained quiescent or divided ≤2 times. In contrast, only 7.3 ± 3.4% of all CFC were derived from these fractions.

for HSC. Brummendorf et al. [31] as well as our group [32] demonstrated asymmetry of cell divisions among hematopoietic progenitors with different growth kinetics of postdivisional subclones. In this context, we reported that the ratio of cells undergoing asymmetric divisions versus dividing cells remained constant and cannot be influenced by cytokine stimulation [31,32,35,36]. Our present data also confirmed that 20% of all $CD34^+CD38^-$ cells divided asymmetrically independent of the media conditions [35,36]. Whereas more than 80% of cells that subsequently yielded ML-IC underwent at least one asymmetric division, only 20% of all cells yielding CFC divided asymmetrically. Thus, these data support the hypothesis that symmetry of division is under intrinsic control and occurs at defined ratios. However, the number of asymmetric divisions increases with the primitiveness of the initially plated cell [31,32,54]. Several reports demonstrated that asymmetric divisional behavior is related to more primitive rather than committed function [31,32, 35,36]. With our unique single cell culture technology, we unequivocally proved that the vast majority of the most primitive progenitors, measured as ML-IC, divided asymmetrically, independent of the extrinsic conditions.

Table 4. Differences between individual ML-IC and CFC based on initial division behavior in LAPM conditions

	ML-IC (n = 6)	CFC (n = 8)
Earliest time point of cell cycle entrance (initial quiescence)	96 hours	48 hours
Q + slow-dividing fraction*	100%	7.3%
Fast-dividing fraction*	0%	92.7%
Asymmetric dML-IC†	5/6	20.2 ± 5.5%

*Slow-dividing cells are defined as cells with ≤2 divisions; fast dividing cells are defined as > 2 divisions during a 10-day culture period.

†dML-IC = dividing ML-IC that could be assessed for division (a)symmetry.

Q = quiescent cells.

This observation is relevant in terms of numeric expansion versus maintenance of primitive function, as one cell with ML-IC capacity that divides symmetrically could produce two or more daughter cells with ML-IC potential. In this scenario, we would have underestimated the ML-IC frequency of the dividing cells. However, our data indicated that 1) the majority of primitive cells produced asymmetrically dividing cells, with only one of two daughter cells possessing the same functional properties as the mother cell; and 2) in 4 of 6 ML-IC that cycled (dML-IC as shown in Fig. 3), progeny of the first- or second-generation daughter cells underwent apoptosis, suggesting that at least in our conditions only maintenance, but not expansion, of primitive cells is possible. This observation is in alignment with previous reports [31,32,53] and demonstrates that this experimental setup allows insights into the maintenance/expansion ratio of proliferated primitive hematopoietic progenitors.

Whether the same progenitor can yield both CFC and primitive LTC-IC (or ML-IC) depending on the extrinsic stimulation has been a controversial issue [55–57]. We clearly demonstrated that they are derived from distinct clones with different cell division kinetics. Studies currently are under way to test our hypothesis by transplanting cell clones, which have been purified based on their divisional history, into xenogeneic in vivo models. These studies will assist in the prospective identification of functional subsets of human hematopoietic progenitors for therapeutic purposes.

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The symmetry of initial divisions of human hematopoietic progenitors is altered only by the cellular microenvironment

Michael Punzel, Daihong Liu*, Tao Zhang, Volker Eckstein, Katrin Miesala, and Anthony D. Ho

Department of Medicine V, University of Heidelberg, Heidelberg, Germany

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Objective. **We examined if cellular elements or adhesive ligands were able to alter asymmetric divisions of $CD34^+/CD38^-$ cells in contrast to soluble factors at a single cell level.**

Materials and Methods. **After single cell deposition onto 96-well plates, cells were cocultured for 10 days with the stem cell supporting cell line AFT024, fibronectin (FN), or bovine serum albumin (BSA). The divisional history was monitored with time-lapse microscopy. Subsequent function for the most primitive cells was assessed using the myeloid-lymphoid-initiating cell (ML-IC) assay. Committed progenitors were measured using colony-forming cells (CFC).**

Results. **Only contact with AFT024 recruited significant numbers of $CD34^+/CD38^-$ cells into cell cycle and increased asymmetric divisions. Although most ML-IC were still identified among cells that have divided fewer than 3 times, a significant number of ML-IC shifted into the fast-dividing fraction after exposure to AFT024. The increase in ML-IC frequency was predominantly due to recruitment of quiescent and slow-dividing cells from the starting population. Increase in CFC activity induced by AFT024 was found only among rapidly dividing cells.**

Conclusions. **For the first time, we have demonstrated that asymmetric divisions can be altered upon exposure with a stem cell–supporting microenvironment. For the primitive subset of cells (ML-IC), this was predominantly due to recruitment into cell cycle and increased rounds of cycling without loss of function. Exposure to AFT024 cells also increased proliferation and asymmetric divisions of committed CFC. Hence direct communication between hematopoietic progenitors with stroma cells is required for maintaining self-renewal potential.** © **2003 International Society for Experimental Hematology. Published by Elsevier Science Inc.**

Hematopoietic stem cells (HSC) are characterized by the dual abilities to self-renew and to differentiate into all hematopoietic lineages. These two features require that HSC undergo divisions with simultaneous generation of progenitors that sustain long-term hematopoiesis and others that produce various progeny cells of the distinct blood lineages [1]. One possibility is that some HSC give rise to functionally equivalent daughter cells identical to the mother by self-renewing divisions, and others generate mature progenies and become lost to the pool. However, at some stage along this development maintenance divisions must occur to produce one daughter cell identical to the mother HSC and the other committed to differentiation [2]. Hence, the two daughter cells from a HSC might initially divide symetrically to replenish the stem cell pool, but at some stage asymmetric divisions must occur which result in different fates of the progeny cells [3].

To follow the relationship between division history and the subsequent functional fate, we recently applied time-lapse microscopy to monitor precisely early cell divisions of purified hematopoietic progenitors in combination with functional assays at a single cell level [4]. From these studies, we have confirmed definitively that asymmetric divisions occurred in strong correlation with stem cell equivalent function: Individual cells that gave rise to the most primitive myeloid-lymphoid-initiating cells (ML-IC) were associated with asymmetric divisions and demonstrated sig-

Offprint requests to: Anthony D. Ho, M.D., Dept. of Medicine V, University of Heidelberg, Hospitalstr. 3, 69115 Heidelberg, Germany; E-mail: anthony_ho@med.uni-heidelberg.de

*The first two authors contributed equally to this work.

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nificantly lower division kinetics than those giving rise to committed colony-forming cells (CFC) [5]. Whereas regulatory molecules were able to induce significant changes in mitotic rate and differential colony formation of committed progenitors, the proportion of cells undergoing asymmetric divisions remained unchanged. This observation supported the notion that growth factors alone could not alter the symmetry of divisions and the self-renewal ability of HSC.

There is considerable evidence that cell-cell interactions play an essential role in regulating the balance between self-renewal and differentiation and subsequently the long-term fate of stem cells [6–11]. Although mechanisms of hematopoietic regulation have been extensively studied, it is not yet defined how intrinsic and extrinsic factors regulate proliferation, self-renewal, and differentiation of HSC. Intrinsic factors are governed by gene expressions, whereas extrinsic factors include humoral determinants such as cytokines as well as cellular interactions with the microenvironment. The bone marrow with extracellular matrix (ECM) proteins, such as fibronectin (FN), laminin, L-selectin, and others, as well as various accessory cells collectively known as "the stroma," constitutes the hematopoietic microenvironment [12,13]. The role of the ECM and its cells for survival and growth of HSC has been demonstrated extensively. In addition, the success of long-term cultures always depends on the presence of such artificial niches in vitro [14–16].

Recently, the cell line AFT024 has been shown to support the self-renewal and differentiation of murine and human HSC for more than 7 weeks [17–20]. Feeder layer consisting of irradiated AFT024 has been demonstrated to resemble an artificial "stem cell niche" [19,21]. It has been shown that more than 1000 informative sequences from a AFT024 cDNA library are enriched for molecules that could interact with HSC. These include cytokines, chemokines, adhesion molecules, proteoglycans, cell surface receptors, and other ECM molecules [22,23].

In this study, we investigated if adhesive ligands or cellular determinants, such as the stem cell–supporting microenvironment of AFT024, are able to alter the proportion of cells undergoing asymmetric cell divisions in relation to its subsequent functional fate. A thorough understanding of these fundamental mechanisms is essential to appreciate the power and the means to influence the long-term fate of human hematopoietic stem cells. We have demonstrated that in contrast to adhesion itself, only contact interactions with AFT024 increased significantly the absolute number of cells that have divided asymmetrically as well as the number of cells giving rise to primitive ML-IC and to committed CFC.

Materials and methods

Cell source and preparation

Human cord blood (CB) was collected from the umbilical cord after informed consent using guidelines approved by the Ethics Committee on the Use of Human Subjects at the University of Heidelberg. Mononuclear cells (MNC) were isolated after centrifugation on Ficoll-Hypaque (BIOCHROM KG, Berlin, Germany). $CD34^+$ cells were enriched with a monoclonal anti-CD34 antibody labeled with magnetic beads on an affinity column (Miltenyi Biotec, Bergisch-Gladbach, Germany).

$CD34^+$-enriched cells were incubated with anti-CD34-phycoerythrin (PE) (Becton-Dickinson, San Jose, CA, USA [BD]) and anti-CD38-APC (BD) and labeled with the membrane dye PKH26 (Sigma, St. Louis, MO, USA). Individual $CD34^+/CD38^-/PKH26^{bright}$ cells were sorted into 96-wells plates (NUNC, Roskilde, Denmark) previously coated with either bovine serum albumin (BSA), FN, or AFT024 feeder layer using the automatic cell depositing unit (ACDU) on a FACS-vantage-SE flow cytometry system equipped with an Apple G3 power computer. To ensure that only a single cell was deposited, the ACDU was set up in a low-event throughput (200–500 events/second).

Assessment of initial cell division

Division cultures. Individual cells cells were cultured for 10 days in Myelocult (Stem Cell Technology, Vancouver, BC, Canada). The medium was supplemented with 2.5 U/mL recombinant human erythropoietin (rhEPO; Roche, Hertfordshire, UK), 10 ng/mL recombinant human interleukin-3 (rhIL-3; Cell Systems, St. Katharinen, Germany), 500 U/mL recombinant human interleukin-6 (rhIL-6), 10 ng/mL recombinant human granulocyte-macrophage colony-stimulating factor (rhGM-CSF), 2.5 ng/mL recombinant human basic fibroblast growth factor (Cell Systems), 10 ng/mL recombinant human insulin-like growth factor-1 (Cell Systems), 50 ng/mL recombinant human stem cell factor (rhSCF; R&D Systems, Wiesbaden, Germany), 1000 U/mL penicillin, and 100 U/mL streptomycin (Gibco, Grand Island, NY, USA) as described previously [5].

The 96-well plates were incubated with 50 μg/mL fibronectin adhesion-promoting peptide (FN, Sigma-Aldrich, Deisenhofen, Germany) in phosphate-buffered saline (PBS) or with PBS containing 1% BSA overnight at room temperature. Alternatively, plates were pre-established with confluent layers of the murine fetal liver feeder AFT024 as described before [23]. After the cells were deposited as single cells, the replication history of each cell was monitored by fluorescence microscopy every 12 to 24 hours for the entire culture period. Previous experiments using time-lapse camera monitoring every 1 to 3 hours have defined precisely the kinetics of division and have demonstrated extensively that the cells did not divide more than once within 12 hours under the experimental conditions [4,5].

Cells were maintained in a humidified atmosphere at 37°C and 5% CO_2 and fed 3 times per week with fresh medium. After 10 days of documentation of the divisional history of each individual cell, the content of each well was transferred onto secondary 96-well plates, for subsequent functional assessment.

Definition of asymmetric divisions. We analyzed asymmetric divisions based on the observation of the PKH-26 dye distribution of each individual cell by time-lapse fluorescence microscopy. Using this method we could easily and precisely define the individual division history of each division step of each sorted cell, even in the presence of AFT024 stromal feeders underneath. We have defined a cell division as asymmetric if one of the first-generation daughter cells did not divide and had kept the fluorescence activity over the observation period while the other first-generation daughter cell divided further.

Asymmetric division (AD) was therefore assessed by the percentage of cells that demonstrated at least one asymmetric division during the time course of 10 days' observation vs the total number of cells deposited. Asymmetric division index (ADI) was defined as the proportion of cells that have divided asymmetrically vs the total number of cells that have divided within the observation period.

Functional assessment. After monitoring the divisional history for each single sorted cell, the entire progeny of each initial cell was transferred in either ML-IC or CFC readouts. Thus, the subsequent functional assessment always refers to the initial cell in the freshly isolated cord blood population sorted at day 0. This approach allows assessment of either ML-IC or CFC function for each individual cell. Statistics as described were performed to obtain functional differences of cells with different divisional history.

Myeloid-lymphoid-initiating cell assay

After the 10-day observation period, cells were transferred into the ML-IC assay (Fig. 1), which has been described extensively in previous reports [5,25]. Briefly, cells were transferred from the division cultures on either BSA, FN, or AFT024 feeders into secondary plates containing confluent irradiated AFT024 feeders. After an additional 2 weeks of proliferation the content of each single well of the secondary plates was harvested with trypsin and divided equally over 4 tertiary 96-well plates in such a manner that 1 out of 4 of each single-cell progeny was deposited in the identical location in the 4 tertiary plates. The 4 tertiary plates had been established with a confluent layer of AFT024 cells, 2 of 4 with lymphoid differentiation medium for the natural killer–initiating cell (NK-IC) readout [24], the other 2 with myeloid differentiation medium for the long-term culture-initiating cell (LTC-IC) readout. Detection of at least 1 LTC-IC and at least 1 NK-IC in the identical location in the tertiary plates was required to consider the initially plated CB $CD34^+/CD38^-$ cell as ML-IC [25].

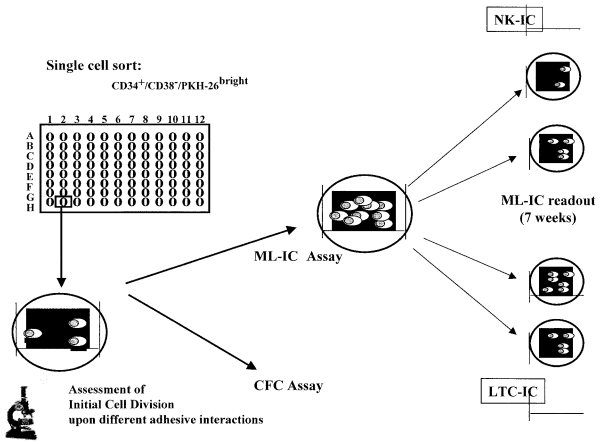

Figure 1. Experimental setup: $CD34^+/CD38^-$ cells were individually sorted onto 96-well plates (1 cell per well) that have been precoated with either BSA or FN or alternatively pre-established with the murine fetal liver feeder AFT024. The division history within the first 10 days of culture was determined by dye-resolution fluorescence microscopy. After the division history for each cell was documented, all progeny of initially sorted cells were transferred to the same location of secondary 96-well plates pre-established with AFT024 to assess myeloid-lymphoid-initiating cells (ML-IC). After 2 weeks of AFT024 culture the content of each well was split into 4 equal parts by volume and equally distributed to the same location of 4 tertiary 96-well plates to assess LTC-IC and NK-IC in a tertiary 7-week culture step. If wells had been tested positive for LTC-IC and NK-IC derived from the same initially sorted cell, this individual progenitor was named as one ML-IC. To assess the CFC frequency, wells of the primary 96-well plates were overlaid with clonogenic methylcellulose medium and scored for colony formation after an additional 12–14 days. In separate experiments ML-IC were detected up front out of the starting population by sorting $CD34^+/CD38^-$ cells directly onto 96-well plates prelayered with the murine fetal liver feeder AFT024.

CFC assay

To determine the CFC frequency after 10 days divisional cultures, the plates were overlaid with clonogeneic methylcellulose medium in a final concentration of 1.12%, supplemented with 30% fetal calf serum (FCS), 2 IU/mL EPO, and supernatant of the bladder carcinoma cell line 5637 and scored for CFC after another two weeks.

Statistics

Results of experimental points from different experiments were reported as mean ± standard error of the mean (SEM). Significance levels were determined by either paired or nonpaired two-sided Student's *t*-test analysis as indicated.

Results

Division kinetics of $CD34^+/CD38^-$ cells upon exposure to soluble and adhesive factors

In 13 different experiments 176 individual $CD34^+/CD38^-$ cells per adherent matrix per experiment were deposited onto 96-well plates that have been precoated with either confluent and irradiated AFT024 feeder layer, FN, or BSA respectively. The divisional history was monitored and scored during a 10-day incubation period. The results are shown in Table 1. When cultured on plates that have been precoated with BSA or with FN, the percentage of quiescent cells was similar (13.8% ± 2.7% on BSA; and 11.9% ± 2.5% on FN; n.s.). When cocultured with irradiated AFT024, only 3.1% ± 0.8% of the $CD34^+/CD38^-$ cells remained quiescent for 10 days ($p < 0.01$ for both comparisons). Thus, more cells were recruited into cell cycle upon contact with AFT024.

Of all the cells that went into cell cycle, 52.0% ± 5.9% of $CD34^+/CD38^-$ cells cultured on BSA and 50.6% ± 5.5% cultured on FN were found in the fraction of cells that have

Table 1. Initial cell division of individual $CD34^+/CD38^-$ cells cultured for 10 days on different adhesive layers

	n	Quiescent	SDF	FDF
BSA	13	13.8% ± 2.7%	22.7% ± 2.3%	52.0% ± 5.9%
FN	13	11.9% ± 2.5%	21.7% ± 2.5%	50.6% ± 5.5%
AFT024	13	3.1% ± 0.8%*	16.2% ± 4.8%	68.2% ± 4.8%†

Values are given as means of relative numbers [%] of cells deposited ± SEM.

*$p < 0.01$ compared to BSA and FN.

†$p < 0.05$ compared to BSA and FN.

SDF = slow-dividing fraction (defined as cells with ≤ 2 divisions); FDF = fast-dividing fraction (defined as cells with > 2 divisions).

undergone more than two rounds of divisions after 10 days, defined as the fast-dividing fraction (FDF). In contrast, 68.2% ± 4.8% of the initially sorted cells cocultured with AFT024 have divided more than two times, hence significantly higher than those cultured on BSA ($p < 0.05$) or on FN ($p = 0.05$). Thus, coculture with the stem cell–supporting microenvironment of AFT024 accelerated the division kinetics of $CD34^+/CD38^-$ cells. The number of cells that died during the 10-day culture period was not significantly different in the three culture conditions (11.5% ± 2.5% in BSA, 15.9% ± 3.3% in FN, and 13.9% ± 1.8% in AFT024 conditions).

Asymmetric division of $CD34^+/CD38^-$ cells upon exposure to soluble and adhesive factors

We examined whether adhesive interactions with the microenvironment could alter asymmetric division behavior. When $CD34^+/CD38^-$ cells were cultured in contact with FN, 22.8% ± 1.9% of the cells underwent asymmetric divisions, similar to cultures without adhesive interactions (22.0% ± 2.4%; n.s.). This observation indicated that adhesion itself did not alter the division asymmetry of $CD34^+/CD38^-$ cells. However, the number of $CD34^+/CD38^-$ cells that have divided asymmetrically increased significantly in cocultures with AFT024 feeder layer (31.0% ± 2.0%) as compared to BSA ($p < 0.01$) or to FN ($p < 0.05$). Exposure to AFT024 also elevated the ADI, i.e., the ratio of cells that has divided asymmetrically to all cells that went into cell cycle as demonstrated in detail in Table 2.

Thus, for the first time we have demonstrated that only contact to the stem cell–supporting microenvironment, AFT024, was able to elevate the absolute number of $CD34^+/CD38^-$ cells that underwent asymmetric divisions as well as the ADI.

Impact of adhesion on functional properties of $CD34^+/CD38^-$ cells with primitive function

We then determined the impact of adhesive interactions on the functional properties of the most primitive $CD34^+/CD38^-$ cells at a single-cell level measured as ML-IC. After monitoring the initial division behavior over a 10-day period in different adhesion cultures for 176 cells per experiment in 4 independent cord blood samples, we transferred the entire progeny of each individual $CD34^+/CD38^-$ cell into the ML-IC assay. Similar numbers of ML-IC could be obtained on either BSA (4.6% ± 1.0%) or FN (6.2% ± 3.2%, n.s.). However, significantly more cells with ML-IC function have emerged after 10 days of coculture with AFT024 (10.5% ± 0.7%; $p < 0.01$ compared to BSA and $p < 0.05$ compared to FN). In addition, when compared to the ML-IC content of freshly sorted $CD34^+/CD38^-$ cord blood progenitors (6.8% ± 0.1% n = 4), contact with AFT024 increased the number of ML-IC per individually sorted cell significantly ($p < 0.05$, nonpaired *t*-test). This suggests that the microenvironment of AFT024 also recruited very primitive and dormant cells into cell cycle that otherwise would have remained quiescent (Fig. 2).

Table 2. Asymmetric division of individual cultured CB $CD34^+CD38^-$ cells on different adhesive layers

	n	Total cell divisions	Asymmetric cell division	Asymmetric division index (ADI)
BSA	13	86.6% ± 2.1%	22.9% ± 1.9%	31.0 ± 2.0
FN	13	87.5% ± 2.1%	22.8% ± 1.9%	31.8 ± 2.5
AFT024	13	96.9% ± 0.8%*	31.1% ± 2.0%†	38.1 ± 2.9‡

Values are given as means of relative numbers [%] of asymmetric dividing cells per cells deposited ± SEM.

*$p < 0.05$ compared to BSA and FN.

†$p < 0.01$ compared to BSA and < 0.05 compared to FN.

‡$p < 0.01$ compared to BSA and $p = 0.1270$ compared to FN.

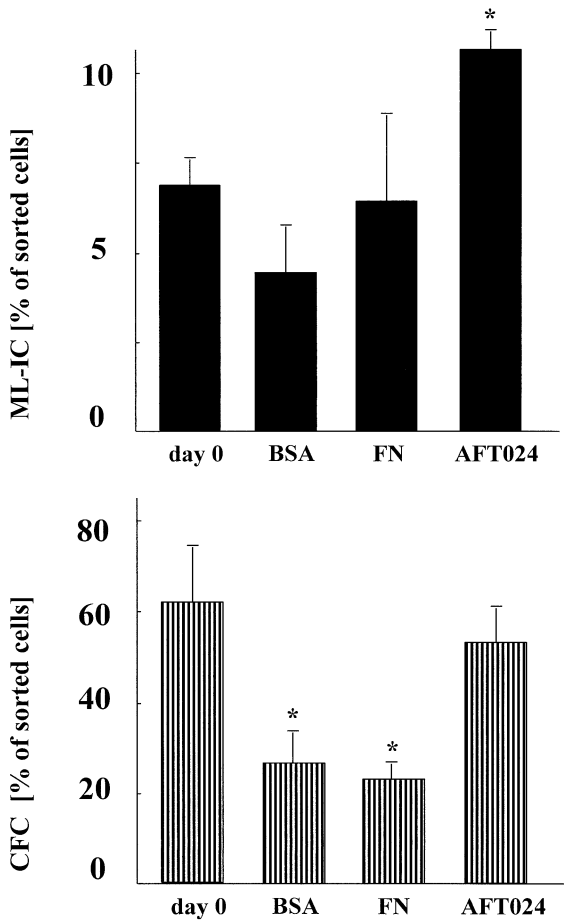

Figure 2. ML-IC and CFC content in relation to the adherent matrix. Cells were individually sorted onto 96-well plates precoated with either BSA or FN or AFT024. The division history was determined by dye-resolution fluorescence microscopy. After 10 days of culture the entire progeny of the single sorted cells were transferred either into the ML-IC assay **(A)** or overlaid with clonogenic methylcellulose to assess CFC **(B)**. To compare progenitor frequency at day 10 with the initial frequency in the freshly sorted population, ML-IC and CFC frequency have been assessed in 4 independent experiments by sorting cells directly into the ML-IC or CFC assay. (*$p < 0.05$ compared to BSA)

This observation was further confirmed by analyzing the division behavior of $CD34^+/CD38^-$ cells that subsequently yielded ML-IC. The results are depicted in Table 3. Upon exposure to BSA or FN, 35.0% ± 6.1% and 39.3% ± 22.9% of ML-IC were found, respectively, among the quiescent fraction, whereas only 9.0% ± 5.9% (n = 4; $p < 0.05$) of the cells with ML-IC capacity have been recruited from the quiescent fraction in cocultures with AFT024. In addition, only after contact with AFT024 feeder layers, individual $CD34^+/CD38^-$ cells that have divided more than two times (FDF) yielded ML-ICs. This again indicated that interaction with AFT024 was able to drive the most primitive cells into cycle.

Of all the $CD34^+/CD38^-$ progenitors with more than two divisions (necessary to assess asymmetric cell division) and subsequent ML-IC function, 65.6% ± 13.8% (n = 4) divided asymmetrically during the initial 10-day coculture with AFT024. This confirmed our previous experiments showing that primitive ML-IC divided mainly asymmetrically regardless of the culture conditions [5]. In cultures with BSA or FN most of the ML-IC were found exclusively among $CD34^+/CD38^-$ cells that have remained quiescent or divided just once. Only one ML-IC was found that underwent more than one round of cell divisions under such conditions and this occurred asymmetrically.

Impact of adhesion on functional properties of $CD34^+/CD38^-$ cells with committed function

Next, we correlated the early division behavior of each individual $CD34^+/CD38^-$cell with subsequent committed function, assessed in the CFC assay. Individual $CD34^+/CD38^-$ cells (176 cells per experiment in 4–6 individual cord blood samples) were sorted singly onto the 96-well plates precoated with BSA, FN, or AFT024 feeder layers. The divisional history was monitored every 12 to 24 hours and after 10 days the entire progeny of each individually sorted cell was replated into clonogeneic methylcellulose and assessed for CFC.

As shown in Figure 2B, similar numbers of CFCs could be obtained in cultures exposed initially to BSA (27.9% ± 3.5%) and FN (23.7% ± 2.4%, n = 6; n.s.), suggesting that adhesion of $CD34^+/CD38^-$ cells to FN did not influence the clonogenic capacity. However, this was significantly less compared to the starting population that contained 62.0% ± 15.7% CFC. In contrast, when $CD34^+/CD38^-$ cells were initially cocultured with AFT024, the frequency of CFC could be maintained compared to day 0 (54.1% ± 5.0%; n = 4; n.s.). This indicated that contact with the AFT024 microenvironment was able to maintain the clonogenic capacity of the $CD34^+/CD38^-$ cells on a cell-per-cell basis.

The relationship between division history and $CD34^+/CD38^-$ cells that subsequently yielded CFC is demonstrated in Table 4. As shown, 92.1% ± 3.5% of all CFCs were derived from the fast-dividing fraction (FDF, cells with more than 2 divisions) when cultured upon BSA-precoated plates; subsequently only 7.9% ± 3.5% of the CFC were recruited from the slow-dividing fraction (SDF, cells with ≤2 divisions). Significantly fewer clonogeneic progenitors (80.3% ± 5.7%) have divided more than two times (FDF, $p < 0.05$ compared to BSA) when initially cultured on FN-coated plates. This indicated that contact with FN decreased specifically the division kinetics of clonogeneic cells that have already been in cycle.

Upon exposure to AFT024 for 10 days, almost all (99.0% ± 0.5%, $p < 0.01$) cells with subsequent CFC function entered cell cycle. The observation among cells exposed to FN and AFT024 indicated that contact to the AFT024 microenvironment could counteract the FN-mediated inhibition of cell cycling.

We then examined the relationship between committed progenitor activity (as reflected by CFC) and initial symmetry of division of the $CD34^+/38^-$ cells. The results are summarized in Table 4. As shown, 21.5% ± 4.5% of all CFCs cultured on BSA have divided asymmetrically, which confirmed our previous observation. In contrast, when cells with subsequent CFC capacity were cultured on FN (n = 6) or AFT024 feeder layer (n = 4), significantly more progenitors have divided asymmetrically (33.6% ± 6.2% on FN, $p < 0.01$ and 36.4% ± 3.0% on AFT024, $p < 0.05$). Thus, contact with AFT024 feeder layer not only induced hematopoietic progenitors to proliferate more extensively but also increased asymmetric divisions of committed progenitors.

Table 3. Initial division of $CD34^+/CD38^-$ cells generating ML-IC after 10 days of culture on different adhesive layers

	Quiescent	SDF	FDF
BSA (n = 4)	35.0% ± 6.1%	65.0% ± 6.1%	0.0% ± 0.0%
FN (n = 4)	39.3% ± 22.9%	60.7% ± 22.9%	0.0% ± 0.0%
AFT (n = 4)	9.0% ± 5.9%*	75.4% ± 11.5%†	15.6% ± 11.8%†

Values are mean of relative numbers [%] per all ML-IC ± SEM.

*$p < 0.05$ compared to BSA, †$p < 0.01$ compared to BSA and FN.

SDF = slow-dividing fraction (defined as cells with ≤2 divisions); FDF = fast-dividing fraction (defined as cells with >2 divisions).

Table 4. Initial cell division of $CD34^+/CD38^-$ cells that subsequently produced colony-forming cells (CFC) after 10 days of culture on different adhesive layers

	n	CFC from FDF	Asymmetric CFC
BSA	6	92.1% ± 3.5%	21.5% ± 4.5%
FN	6	80.3% ± 5.7%*	32.5% ± 5.9%*
AFT024	4	99.0% ± 0.7%†	36.4% ± 3.0%*,‡

Values are mean of relative numbers [%] per all CFC ± SEM.

*$p < 0.05$ paired *t*-test compated to BSA.

†$p < 0.05$ nonpaired *t*-test compared to FN.

‡$p < 0.05$ nonpaired *t*-test compared to BSA.

FDF = fast-dividing fraction (defined as cells with >2 divisions); Asymmetric CFC = asymmetric divided CFC/total CFC.

In addition, exposure to FN decreased the division kinetics of cycling progenitors and relatively enhanced asymmetric divisions of functionally committed progenitors.

Division kinetics and subsequent functions

To illustrate the relationship between cell division kinetics and subsequent function, we have correlated the division history of $CD34^+/CD38^-$ cells and their subsequent functions in producing ML-IC or CFC. The results are summarized in Figure 3. Upon exposure to BSA for the first 10 days, ML-IC were exclusively found among $CD34^+/CD38^-$ cells that have divided fewer than three times, as previously demonstrated (Fig. 3A). Preincubation with FN yielded similar results. Upon coculture with AFT024, different division kinetics of $CD34^+/CD38^-$ cells yielding ML-IC were evident. Although most of the ML-IC (75.4% ± 11.5%) were still identified among cells that have divided fewer than three times, 15.6% ± 11.8% of the ML-IC were in the FDF, vs 0% when incubated with BSA or FN.

The relationship between division history and $CD34^+/CD38^-$ cells that subsequently generated CFC is demonstrated in Figure 3B. Whereas CFC were found in increasing numbers with every division upon incubation with BSA, almost all of the CFC (99.9% ± 16.9%) were found among $CD34^+/CD38^-$ cells that have divided rapidly (>3 divisions) when cocultured with AFT024. Slightly more CFC were identified among quiescent $CD34^+/CD38^-$ cells incubated with FN and less upon every cell division as compared to cultures with BSA, except those that have divided two times. This observation also indicated that other determinants have been activated by contact with AFT024 that overrode the FN-mediated inhibition of cell cycling.

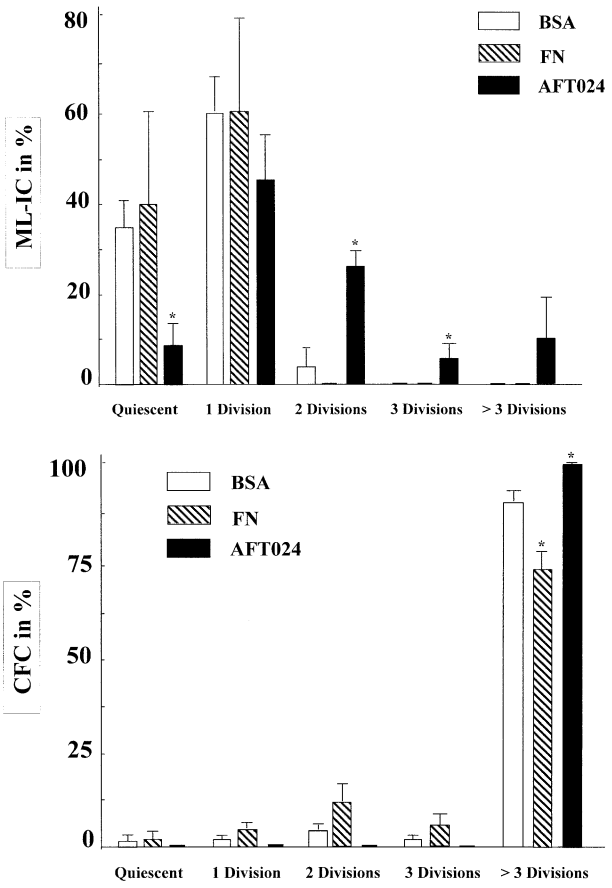

Figure 3. Only contact with AFT024 increases the proliferative status of primitive ML-IC and committed CFC. Individual $CD34^+/CD38^-$ cells were sorted onto 96-well plates (1 cell per well) and cell division was determined by dye-resolution fluorescence microscopy. After 10 days of culture, the entire progeny of each individual well was transferred into the ML-IC assay to correlate primitive function with the divisional history for each single cell **(A)**. In different plates, cells were overlaid with clonogenic methylcellulose **(B)** to assess the formation of CFC. Contact with AFT024 not only recruited more primitive ML-IC into cycle but also shifted the proliferative status of a significant proportion of ML-IC into 2 and more rounds of cycling. Contact with AFT024 also increased the proliferation of CFC **(B)** significantly up to 100% fast-dividing CFC (>2 divisions). (*$p <$ 0.05 compared to BSA)

Discussion

For the first time we have demonstrated that contact of primitive $CD34^+/CD38^-$ cells with a stem cell–supporting microenvironment (AFT024) increased asymmetric divisions of both primitive and committed progenitors by recruiting significant numbers of primitive cells into cell cycle. This phenomenon of recruitment as well as the shift in asymmetric division could not be induced by cytokines alone or in combination, as demonstrated previously [4,5]. The increase in ML-IC frequency was found in the starting population that has divided during the 10-day period upon exposure to AFT024. This observation indicated that dormant cells that are usually in G_0 can be recruited to cycle without loss of primitive function after cross-talk with AFT024, whereas upon exposure to BSA or FN, ML-IC were found almost exclusively among quiescent cells or cells that have divided only once within 10 days.

Earlier studies have demonstrated that the most primitive human or murine precursors are quiescent or have very slow cycling rates independent of cytokine exposure or regulatory molecules [26–30]. Whereas most of the evidence was derived from bulk culture experiments, our group was able to demonstrate this at a single cell level. Using our single-cell-culture technology we have previously demonstrated that primitive ML-IC were recruited exclusively from either cytokine-nonresponsive (quiescent) or slowly dividing cells. Due to the quiescence of cells yielding ML-IC, asymmetric divisions could not always be related to primitive function in general [5]. However, if ML-IC did divide, this occurred asymmetrically [5]. The present study provided the first evidence that only contact with a stem cell–supporting microenvironment was able to increase the absolute number of cells undergoing asymmetric divisions. This was observed in the subsets of cells that yielded both primitive (ML-IC) and committed progenitors (CFC). It is important to note that

our experimental approach examined the entire progeny of an initially sorted cell and therefore related only to one individual cell at day 0. Thus, it can be argued that one cell can make both ML-IC and CFC from the two first-generation daughter cells, which could be considered as a functional asymmetric division. However, we have already demonstrated that single $CD34^+/CD38^-$ cells and subsequently its entire progeny were distinct in their functional property for either ML-IC or CFC function at day 0. This could be unequivocally identified by its initial divisional behavior [5].

The hallmark of a stem cell is the dual ability to self-renew and to differentiate into progenitors of multiple lineages. The two daughter cells from a HSC might be initially equivalent to replenish the stem cell pool, but subsequent cell divisions must result in different fates of the progeny cells. Alternatively, a balance between symmetrical cell divisions that result in self-renewal vs those which result in differentiation might be able to maintain the stem cell pool and provide a source of multipotent progenitors [1]. In this model a subset of HSC gives rise to functionally equivalent daughter cells identical to the mother cell by self-renewing divisions, and another subset generates mature and equivalent progenies that become lost to the pool. Considering all the various possibilities between these two models, it is still surprising that most studies provide evidence for self-maintaining asymmetric divisions and for the association between asymmetric division with primitive function in ex vivo conditions [4,5,30,31]. Confirming these previous results, the present report indicates that the increase in primitive and committed progenitors upon AFT024 cocultures is mainly induced by the recruitment of dormant cells from the G_0 phase (ML-IC) or cell-cycle enhancement (CFC). Although the possibility could not be excluded that self-renewing symmetric divisions occurred upon contact with AFT024, this was not likely, as the increase in ML-IC or CFC was invariably accompanied by an increase in asymmetric divisions. Furthermore, recent experiments from our group demonstrated that under in vitro conditions, the primitive ML-IC function is asymmetrically distributed to only one of the two first-generation daughter cells after initial division (manuscript in preparation). This observation supports the hypothesis that divisional symmetry would be intrinsically determined if only cytokines were involved, as shown by other reports [4,5,30–35]. The increase in asymmetric divisions upon coculture with AFT024 suggests additional regulation of the division program by cellular determinants. Moreover, our data indicated that in vitro maintenance and/or expansion of the most primitive stem cells was made possible only through recruitment of quiescent cells by coculturing with AFT024.

Of paramount interest is the identification of factors in the AFT024 microenvironment that are responsible for these effects. Using a subtractive hybridization approach between AFT024 and nonsupportive stromal cell lines such as 2018, Moore et al. have identified more than 1000 factors that could be responsible for the supportive effects of AFT024 [22]. Among such candidates were notch-ligands, such as mouse dll-1, as well as specific large-size 6-O-sulfated glycosaminoglycans [21,36,37].

It has been reported that adhesion of hematopoietic progenitors through engagement of $\alpha4\beta1$ integrins to FN is accompanied by proliferation inhibition, which is associated with elevated levels of $p27^{KIP}$, a contact-dependent cyclin kinase inhibitior [38,39]. This inhibition of proliferation may preserve HSC by preventing differentiating cell divisions [40] or increased survival [41]. However, depending on the experimental conditions it has been shown that decreased proliferation may also be responsible for decreased progenitor maintenance [8,38,42]. In our single-cell system we did not observe any effect of FN on the most primitive progenitor subset as suggested by others [40,41,43]. However, upon exposure to FN, the number of cell cycles that the committed progenitors underwent during the observation period decreased. This was accompanied by a relative increase of asymmetric divisions. As we and others reported previously, FN-mediated signaling might be distinctly different in committed vs primitive progenitor subsets [38,44–46]. Thus, a plethora of factors provided by the microenvironment might be essential in the regulation of self-renewal vs differentiation as well as cell numbers.

Recently, studies of transdifferentiation in rodent models also indicated that surrounding cells in the microenvironment play a major role in defining the long-term fate of an adult stem cell. Transplanted HSC from an allograft, once homed to the preinjured organ, are probably "educated" by the neighboring cells to maintain a balance between self-renewal and differentiation [11]. The specific supportive cells in the stroma are then responsible for stem cell renewal, coupled with opposing signals from other accessory cells that promote differentiation of daughter cells displaced outside the stem cell niche [47,48].

Kiger et al. [49] and Tulina and Matunis [50] have recently defined the molecular nature and spatial organization of the signaling pathway that governs stem cell fate in the *Drosophila* testis. Upon division of a germline stem cell, the daughter cell in direct contact with the hub retains the self-renewal potential, whereas the other daughter cell was destined to differentiate into a gonioblast and subsequently into spermatogonia. Both reports provide evidence that *unpaired*, a ligand activating the JAK-STAT signaling cascade, expressed by the apical hub cells in the testis, causes stem cells to retain self-renewal potential. Analogous to this finding, our observation demonstrated that direct communication between HSC with the stroma is required for maintaining and expanding the self-renewal potential of HSC. Further research is necessary to define how the functional cross-talk between HSC and the niche occurs, how this education will define the differentiation pathway, and how the balance between self-renewal and differentiation is regulated.

Although we are yet far from an understanding of the mechanisms leading to regulation of self-renewal vs differ-

entiation, our technology has provided evidence for the role of contact of human HSC with a functional microenvironment for the division behavior and long-term fate.

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