に実務担当者がしていることを確認する，あるいはその報告を受けているのではない。プロジェクトで何をしなければならないのかを確認し，担当者の役割分担を決め，担当者の動きを素早くキャッチして，プロジェクトを最短距離で進め，またレールから脱線しないように積極的に仕掛けていくことである。臨床研究では，以下に述べる作業を広く理解し，状況判断しながら確実に前に進めていくことが重要である。なお，プロジェクトマネジメントは研究者自身が行うことも可能であるが，プロジェクトマネジャーにできるところは任せ，研究者は研究に専念したい。ここではプロジェクトマネジャーが存在することを前提に話を進める。

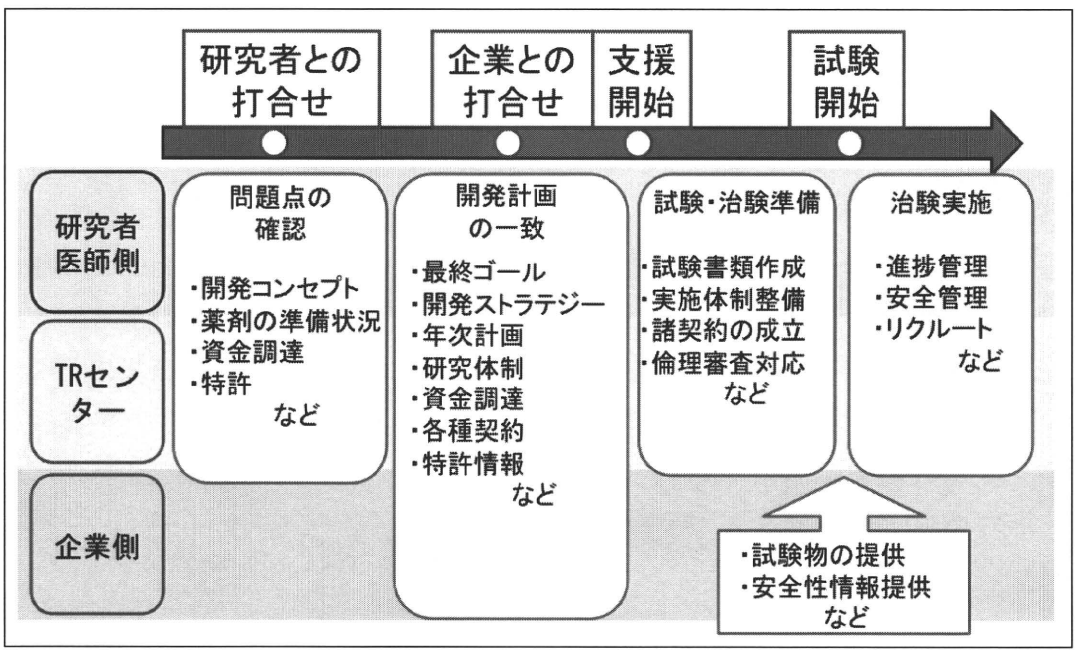
Ⅱ．プロジェクトマネジメントの業務

1．臨床研究開始前（図❶）

(1) 研究者側との調整

研究者からのシーズ支援の相談があった場合には，まず研究者とテーマについて十分な時間をかけて議論する。どのようなゴールをめざすのか，企業の協力は得られているのか，資金準備は大丈夫なのかなどである。そしてゴールへ向かうために最短ルートを模索する。開発ロードマップなどの年次計画を立ててみると，そこで初めて研究者は臨床研究がいかに長期戦であるかを理解する。たいていの研究者は自身がどれだけのことをしていかなければならないのか十分把握しないまま臨床研究を始める場

図❶　臨床研究が開始するまでの主な流れ



合が多い（とりあえず，始めてから考える）ので，現実を話し合い，研究者が十分納得したうえで研究を進めることが非常に重要である。実際に行う作業としては，試験デザイン，法令遵守，被験者の安全性，データの質の担保，標準業務手順書の理解などであり，研究者に一つ一つ理解してもらい，今後の作業を十分に理解してもらう。また，忘れずに資金調達に関しても議論しておく必要がある。公的資金であれば時限つきであるため，研究計画立案の際に期間や症例数などの研究規模を含めて検討しなければならない。もし，企業とあらかじめ連携している場合には，プロジェクトマネジャーは企業側と研究者を交えて早急に折衝をしてゴールを議論する。最終的に開発するものが医薬品や医療機器であれば，企業側が臨床研究を引き続くこととなるため，どのような研究成果を期待し，どのように活用するかを最初の時点で議論しておくことは先々を考えると非常によいことである。臨床研究は，単なる研究ではなく，しっかりとしたプロジェクトとして進めることを認識する必要がある。

(2) 臨床トラックは何か

臨床研究を始めるにあたっては，臨床研究の流れを十分に理解しておく必要がある。そして研究者側へ説明し，プロジェクトの方向性を決定する。単なる臨床研究であれば，「臨床研究に関する倫理指針」に則って実施することとなる。研究者はその指針に基づいて先ずはプロトコルを作成する必要がある。したがって，当該指針の研究者への説明と理解は必要である。2009 年 4 月から改訂された当該指針では，新たな事項も盛り込まれ，特に臨床研究の実施の際の補償保険への加入も謳われている。プロトコルなどが無事に完成すれば，倫理審査委員会への提出になる。倫理審査委員会からは後日意見が来るため，その意見に対する回答作成が必要となる。承認が得られれば，実施に向けた医療機関内関係部署との打合せを進める。また経費について医療機関とあらかじめ折衝する必要があるものは解決させておく。このように実施までにかかる作業から時間をあらかじめ計算し，研究者および関係者と情報を共有する。スケジュールが遅れるようであれば，早期に関係者へ情報を提供する。以上の内容が医療機関で行う臨床研究のほぼスタンダードな流れである。

さらに，臨床研究の先には，国（厚生労働省）が設置した，より臨床応用を意識した制度である「先進医療」や「高度医療」も存在している。医療機関の倫理審査を終えた後にそれらの制度を利用した臨床研究をめざすことも可能である。もしその場合には，先進医療では実施医療機関での実施例や有効性を謳うエビデンスが必要であり，先進医療に係る具体的な経費算出も必要である。それらをまとめたうえで厚生労働省への提出・承認が必要である。先進医療はいったん認められると症例数がある程度蓄積すれば保険医療としての承認が見えてくる。一方，高度医療の場合には，先進医療よりもまだ研究段階のものを対象としており，プロトコルなどの資料や高度医療にか

かる経費算出などを行ったうえで，これも厚生労働省への提出・承認が必要である。また高度医療は試験的な要素が強く，結果は次の治験などのエビデンスとして活用できる。これら両制度ともに一般医療に向けたトラックとして有望である。

もう1つのトラックとしては，「治験」が存在する。治験の場合は，基本的にGCPに遵守することになるため，データの質の担保の観点から，モニタリングや監査などを実施しなければならない。さらに治験では膨大な資料を作成する必要がある。なお，治験実施には臨床研究と同様に医療機関の倫理審査委員会の承認が必要となる。その資料作成，意見に対する回答などの対応をしなければならない。承認が得られてもすぐに実施できることではなく，治験計画届書を規制当局へ提出する必要がある。企業が行う治験であれば，資料作成や経費は企業側が負担することになるが，医師主導治験では研究者が資料準備から資金準備まですべてしなければならず，日常診療を抱える医師のみの実施はほぼ不可能である。また治験にかかる費用と労力は通常の臨床研究とは違い，かなりハードルが高い。しかしながら，治験の考えは三極（米国，欧州，日本）で同じであることから，いったん苦労して得られた成績やエビデンスは国内外での承認申請の評価資料にも十分活用できる。

(3) 企業との折衝

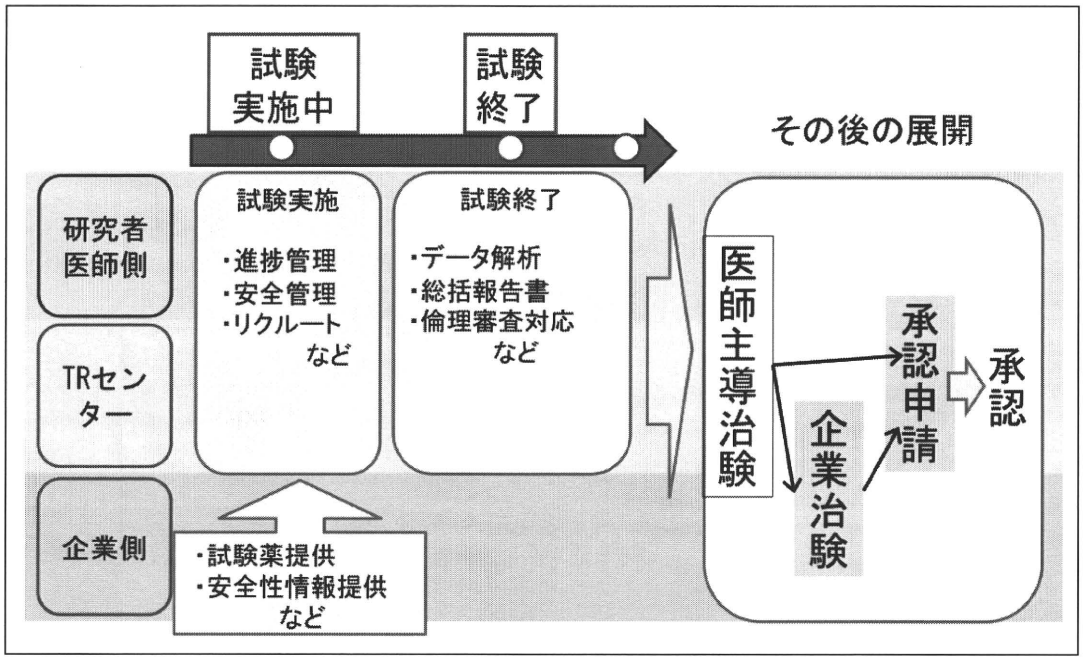
企業と連携して臨床研究を実施する場合には注意が必要である。企業が未承認の医薬品や医療機器を臨床研究に提供する場合には，薬事法に抵触することもあり，ガイドラインなどをよく理解する。企業との共同研究が可能となれば契約をまとめる。契約内容に関して，研究者が苦手とすることが多いため，プロジェクトマネジャーを含めて研究テーマのゴールから十分想定して必要なところは議論する。特に注意が必要なのは，得られた成果の取り扱いである。事前に十分な議論をしたうえで契約をまとめる。なお，研究費や成果報酬についても議論をしておきたい。また契約に関しても締結する必要がある。サンプルに関しては納入時期，納入量，保管条件などを企業と調整し，試験物保管管理部署などの関係部署へ伝える。さらに企業が保有する特許についても確認し，今後の臨床研究の際に得られる特許に十分な調整をしておく。

(4) ゴールを見据えた開発ロードマップの作成（図❷）

研究の位置づけからどのトラックを使ってゴールをめざすのがよいか，非常に大事な岐路である。年単位でのスケジュールやプロジェクト経費について確認する。この理解が得られないかぎりはどんな臨床研究であれ，成功できない。

1つの臨床研究を実施する場合には，試験の準備～実施～終了までの実施項目など考えうるものすべてを挙げ，その実施時期と作業時間を考慮してスケジュール表を作成することが重要である。また将来的な開発ロードマップは，臨床研究後の開発方針を企業とも打ち合わせ，一般医療化までの全体の開発ストラテジーの中で，臨床試験

図❷　臨床研究が終了するまでの主な流れ



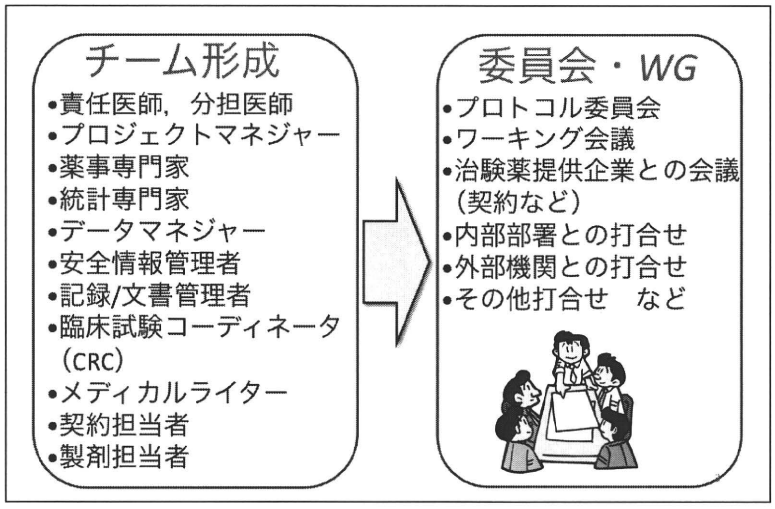
の位置づけを明確にしていく必要がある。多くの議論を踏まえ，この段階になれば臨床研究の位置づけが明確になり，具体的にすべきことに進む。

2. 臨床研究の準備開始

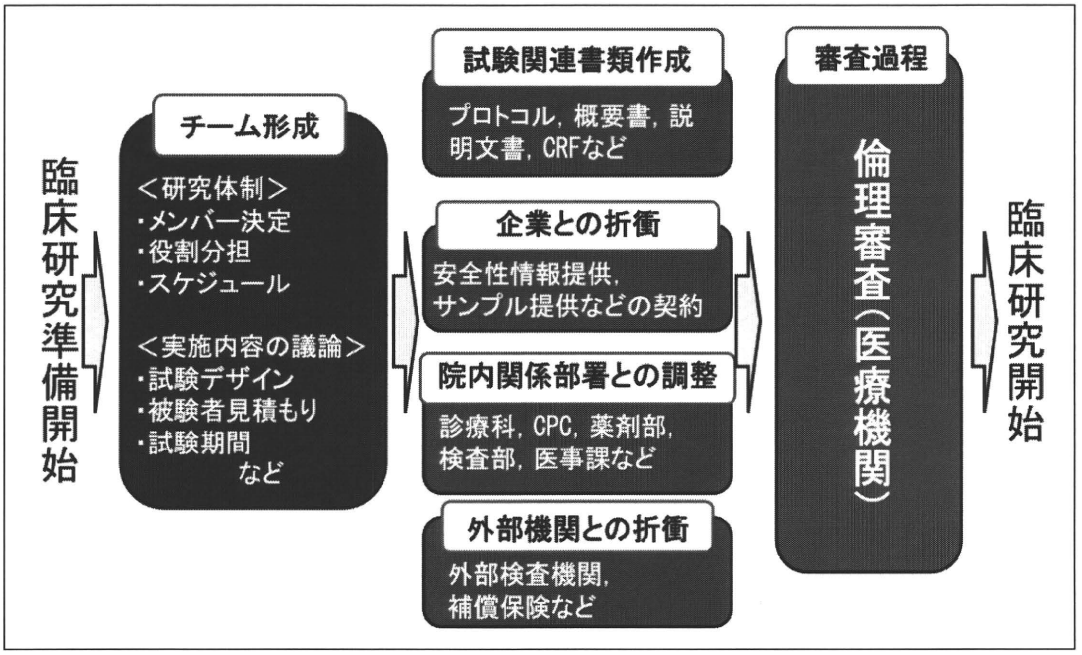
(1) 臨床研究のチーム形成と進捗管理（図❸，❹）

プロジェクトマネジャーはゴールや目標が決まれば，次に支援組織と研究者側を交えて，具体的な今後のスケジュールや実行項目などを議論し，担当者を決定する。そして担当者を中心にプロトコル，説明文書・同意書，試験物概要書などの作成作業を

図❸　臨床研究の準備に必要なチーム・会議



図❹　臨床研究準備のフローと作業内容



進める。プロジェクトマネジャーは実務者会議などを調整し，担当者の負荷を考慮してスケジュールを組み進捗管理をする。その際に，各担当者の動きを十分キャッチする。場合によっては，担当者と個々に打ち合わせて，遅れそうな部分には素早くテコ入れをしてスケジュールの大幅な遅れを未然に防ぐことも必要である。プロジェクトマネジャーは不測の事態などで全体スケジュールに影響する場合には，担当者に早急に知らせ，新たにスケジュールを組み直し，関係者の確認をとる。プロジェクトは1人や少人数で行っていないので，関係者への連絡，相談などの配慮は絶対に必要である。実務担当チームは，責任医師，薬事専門家，統計専門家，データマネジャー，安全情報管理者，臨床研究コーディネーター，契約担当者などから構成される。

(2) 臨床研究に必要な資料

必要な資料はプロトコル作成，概要書作成，説明文書作成，重篤な有害事象発生時のマニュアル，各種マニュアル（効果安全性評価委員会マニュアル，試験物の保管管理マニュアルなど）である。

(3) 規制当局との折衝

臨床研究は，様々な指針やガイドラインにより規定されている。単なる臨床研究の場合であれば，該当する指針に従って試験実施計画を立て，医療機関の倫理委員会や医療機関の長の実施許可が得られれば，研究は開始可能である。研究の目的によっては，規制当局の許可が必要な研究もあり，倫理委員会との調整が先ず必要である。「治験」であれば，医薬品や医療機器の承認申請をめざすものであり，医薬品医療機器総

合機構（PMDA）との折衝は必要不可欠である。PMDAとの対面助言でどのようなことを相談するのか，相談資料の準備も別途必要となる。対面助言の実施前には，対面助言での資料や当日のやり取りなどを打合せする場（事前面談）があり，事前準備として活用する。薬事相談制度の詳細に関しては，別稿にて説明があり省略する。無事に対面助言が終了し，医療機関内のIRBにて承認が得られれば，治験計画届書を提出し，治験が開始となる。

「高度医療」，「ヒト幹細胞を用いる臨床研究に関する倫理指針」などに従った臨床研究であれば，厚生労働省との折衝が必要である。高度医療では事前相談の制度があるため活用する。一方，「先進医療」には直接厚生労働省と折衝する相談制度などはなく，申請書類の作成後に直接提出となる。また「先進医療」や「高度医療」などは医療機関内で経費などの審査を経なければならないので，所定の手続きを踏まえて申請する。プロジェクトマネジャーは規制当局との折衝状況を睨みながら，進捗管理する。

(4) 被験者の見積もり

試験デザインが決定し，プロトコルもほぼ固定できる段階になれば，対象患者も明確になってくる。実際に試験が始まると被験者リクルートに苦労することが多く，プロトコル作成段階から組み入れられそうな被験者を調査する。可能であれば患者一覧を作成し，試験期間中は常に最新の情報にアップすることに努める。また試験が単施設か多施設かで試験の進捗は大きく変わる。単純に考えても単施設で多くの症例を組み入れることは非常に困難である。1症例あたりの試験期間にもよるが，臨床試験は被験者の組み入れが進まなければ試験期間が延長となるため，試験終了の見込みができず，被験者リクルートは失敗の要因にもなる。したがって，プロトコル作成段階から被験者見積もりと患者一覧の作成などは早めの対応が非常に重要な意味をもつ。

(5) 倫理審査

プロトコルや説明文書，概要書などが準備でき規制当局との折衝も折り合いがつけば，いよいよ倫理審査となる。医療機関などでは実施体制は様々と考えられ，呼び方も「セントラル審査」，「IRB」，「倫理委員会」など様々である。申請方法や結果までの過程，かかる日数などを確認しておく必要がある。基本的には研究者側で申請書作成や意見に対する回答作成などを行うが，早急な対応が必要な場合には関係者が支援するような調整は必要となる。なお，倫理委員会とは別で，利益相反委員会の審査も必要な場合もあるため，医療機関内の審査は何があって，どれくらいの期間がかかるのかをプロジェクトマネジャーは確認しておく必要がある。

(6) 医療機関内の関係部署の調整

臨床研究の際には，診療科（手術部やICUなどを含む）の協力は不可欠なため，

試験計画がまとまった段階で事前に説明する。また，試験物が医薬品や医療機器などであれば，研究者とは別の第三者が保管管理をすることが望ましいため，試料保管担当部署（治験管理センターなど）が保管可能かを調整する。大型の医療機器などは保管場所や設置場所を別途調整する必要がある。さらに，臨床研究にかかる経費は非常に大事な問題であり，試験が開始される前に経費関係の事務部門を調整すべきである。特に公的資金を使用する場合には制限があり，拠出元との事前の確認は必要である。最近では，細胞製剤など院内製剤を用いた臨床試験も医療機関にて実施されるようになってきたため，医療機関の試験物製造施設とも十分な打合せが必要である。特に試験物製造施設にとっては，使用時期，経費，マンパワーなど課題があるため研究者とともに協力が得られるよう調整する。

(7) その他

臨床研究は非常に経費のかかる研究であることから，経費の有無は試験の進捗に大きく影響する。なかには研究者側が臨床研究を実施するための十分な資金がない場合もあるため，本来はプロジェクトマネジメントの業務の範囲ではないが，科研費などの公的資金獲得や企業との契約折衝の支援にも関わることが必要になる。また臨床研究では，「臨床研究に関する倫理指針」に従い，補償保険が設計できるのであれば，被験者の安全性の観点から必要である。さらに臨床研究では，特にヒトを対象とする初めての研究であることが多いため，安全性を考慮する必要があり，研究者側とは独立した第三者的な評価委員会が必要である。効果安全性評価委員会の設置も必要である。

これら実施内容が十分準備できれば，いよいよ臨床研究が開始できる。

3. 臨床研究の実施中

(1) 被験者リクルート

被験者の見積もりに沿ってリクルートの進捗を確認する。試験の成功の鍵は被験者リクルートをいかに行うかといっても過言ではない。リクルートが滞った場合には，プロジェクトマネジャーは関係者と対策を早急に協議する。例えば，参加医療機関を増加するなどが挙げられる。

(2) 有害事象発生時の対応

特に重篤な有害事象が発生した場合には，医師が中心となり，あらかじめ策定したマニュアルに従って被験者の保護や医療機関の長などの関係者への報告を行い，迅速な対応をする。場合によっては，プロジェクトマネジャーは早急に研究者とも打ち合わせ，研究の中断・中止を判断する。

(3) 倫理委員会への報告

研究の進行中も倫理委員会への報告事項があるため，研究者側は定期的に報告する。また計画書や説明文書など変更があれば，変更申請を行う。なお，「先進医療」，「高

度医療」などは厚生労働省へ，「治験」などはPMDAへ，報告義務がある内容についてはその手続に従って行う。

4. 臨床研究の終了

(1) 研究のまとめ

研究が目標症例数に達成し観察期間など試験期間が終了した場合，あるいは中止した場合には，研究のまとめ作業となる。プロジェクトマネジャーは研究の開始の際と同様に，データマネジメント，統計解析，報告書作成など研究者と今後のスケジュールを立てて関係者と作業を打ち合わせる。また終了に際しての院内関係部署への確認は必要である。研究の終了の際には，倫理委員会などへの最終報告を行う。「先進医療」や「高度医療」であれば終了報告を厚生労働省へ，「治験」であれば治験終了届書をPMDAへ提出する。最終的な結果は，研究者が論文としてまとめ公表する。

(2) 企業への継承

企業と連携しているプロジェクトであれば，プロジェクトマネジャーは研究が終了に近づいてきた段階で打合せを行い，ゴールの達成や今後の動きを具体的に議論する。開始前に十分な議論が企業との間に行われていれば，研究のまとめ作業が終了し企業へスムーズな移管を行う。

5. 最終的なゴールへ

臨床研究の最終的なゴールは，一般化医療へもっていくことにある。臨床研究の中でのプロジェクトマネジメントとは，研究目的を達成し，企業への継承後も企業と一般化までの道筋を一緒に考え，引き続き協力をしていくことも役目の1つと考える。もし，共同研究した企業と新たなプロジェクトを開始する場合にも，これらの経験は十分生かせることになり，信頼関係も構築できると考える。いろいろな意味で次にも生かせる研究があってこそ，臨床研究である。

おわりに

臨床研究は，実に長い物語である。基礎研究のように実験を次々として結果が出てくるようなものではなく，ヒトを対象としているため安全性や臨床効果の面で十分練ったうえで実施されるものであり，年単位での試験である。まずそこを理解しなければ臨床研究は進まない。また，十分熟慮したとしても，必ずしも成功するとは限らない。しかし努力しなければ，必ず失敗する。

プロジェクトマネジャー自身は，研究者をはじめ多くの関係者へ能動的に働きかけをする必要がある。決して受動的になってはプロジェクトは前進しない。臨床研究の成功の鍵は，関係者をその気にさせ，いかにプロジェクトを前へ動かそうとするかが

ポイントである。プロジェクトマネジメント業務は形のないものを形にする実にタフな業務ではある。

最後に，プロジェクトマネジメントとは，時間と資金とマンパワーのバランスをとって，プロジェクトが一番早くゴールに辿り着くことをサポートすることである。

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  http://www.tr.mext.go.jp/

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Cross-priming of CD8$^+$ T cells in vivo by dendritic cells pulsed with autologous apoptotic leukemic cells in immunotherapy for elderly patients with acute myeloid leukemia

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Objective. **The prognosis for elderly patients with acute myeloid leukemia (AML) remains dismal. To explore the potential of immunotherapy for improving clinical outcomes for these patients, we performed a phase I clinical trial of dendritic cell (DC)–based immunotherapy for elderly patients with AML.**

Materials and Methods. **Autologus monocytes were obtained after reducing tumor burden by chemotherapy. Immature DCs induced with granulocyte-macrophage colony-stimulating factor and interleukin-4 were pulsed with autologous apoptotic leukemic cells as antigens. DCs were administered intradermally to four patients five times at 2-week intervals. To facilitate DC migration to lymph nodes, injection sites were pretreated with killed *Streptococcus pyogenes* OK-432 one day before. DCs were coinjected with OK-432 to induce maturation and interleukin-12 production in vivo.**

Results. **Antileukemic responses were observed by an interferon-γ enzyme-linked immunospot assay or a tetramer assay in two of four patients. In a human leukocyte antigen–A*2402-positive patient, induction of CD8$^+$ T-cell responses to WT1- and human telomerase reverse transcriptase–derived peptides were observed, indicating cross-priming in vivo. The two patients with antileukemic immunity showed longer periods of disease stabilization than the other two patients.**

Conclusions. **This study demonstrates the immunogenicity of autologous DCs that cross-present leukemia-associated antigens from autologous apoptotic leukemic cells in vivo in elderly patients with AML.**

Management of elderly patients with acute myeloid leukemia (AML) remains a challenge because of a high rate of therapy-related mortality and chemotherapy resistance [1]. Antigen-specific immunotherapy, which is less toxic and kills leukemic cells through different mechanisms than chemotherapy, has the potential capacity to improve the clinical outcomes of these patients. Recent identification of several leukemia-associated antigens prompted us to develop immunotherapy for elderly patients with AML [2].

Active immunization by peptide vaccines can induce antileukemic immunity and clinical responses in AML [3–6]. Clinical trials of dendritic cell (DC)–based immunotherapy for AML have also been reported [7–12]. However, the trial using leukemic cell–derived DCs showed that the generation of leukemic cell–derived DCs was feasible in only a limited number of patients, and even in vaccinated patients the treatment could not induce clinical responses [9]. This may be due to lower immunostimulatory activity of leukemic cell–derived DCs than monocyte-derived DCs (MoDCs) [13]. Recently, the efficient generation of MoDCs from patients with AML has been demonstrated in vitro [14], providing a rationale for the use of MoDCs in immunotherapy for AML.

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There are several parameters to enhance the immunogenicity of MoDC vaccines. Whereas monocytes are cultured with granulocyte-macrophage colony-stimulating factor (GM-CSF) and interleukin (IL)-4 conventionally for 5 to 7 days to induce DCs, a shorter period of culture is sufficient to induce equivalently potent DCs [15]. Among DC maturation–inducing factors, microbial components that trigger the production of IL-12 are beneficial to induce effective adaptive immunity [16]. An extended period of stimulation with microbial components results in DC exhaustion in which DCs lose the capacity to produce IL-12 [17]. Thus, a short-term stimulation can generate optimal DCs that retain IL-12 production. Inflammation in the skin before DC injection facilitates DC migration to draining lymph nodes, leading to a stronger immune response [18,19]. Using apoptotic whole tumor cells as antigens may be instrumental in inducing multivalent immune responses [20].

We performed in vitro assays to optimize these parameters. Based on the results of these assays, we conducted a phase I clinical trial of immunotherapy for elderly patients with AML at the second or later remission setting, using DCs loaded with autologous apoptotic leukemic cells. The treatment was well-tolerated and safe and induced antileukemic immunity in two of four patients, which was associated with transient disease stabilization. Importantly, in one patient, cross-priming of leukemia antigen–specific $CD8^+$ T cells in vivo was explicitly demonstrated. This study indicates the safety and immunogenicity of immunotherapy using MoDCs that cross-present leukemic cell antigens in elderly patients with AML.

Materials and methods

Generation, maturation, and cryopreservation of DCs for in vitro assays

Peripheral blood mononuclear cells (PBMCs) were obtained from healthy volunteers by density gradient centrifugation using Lympholyte H (Cedarlane, Ontario, Canada). Monocytes were purified using anti–CD14-conjugated microbeads (Miltenyi Biotec, Bergisch Gladbach, Germany), or enriched by plastic adherence by incubating PBMCs at 37°C for 2 hours and removing nonadherent cells by pipetting. Monocytes were cultured with 800 IU/mL GM-CSF (Primmune, Kobe, Japan) and 500 IU/mL IL-4 (Primmune) in CellGro DC medium (CellGenix Technologie Transfer, Freiburg, Germany) for 3 days (3d-DCs) or 6 days (6d-DCs). In some experiments, 3d-DCs were frozen in CP-1 freezing medium (Kyokuto Pharmaceutical Industrial, Tokyo, Japan). CP-1 contains 12% hydroxymethyl starch and 10% dimethyl sulfoxide in normal saline and was mixed with 8% human serum albumin before use. DCs were matured with 0.1 KE/mL OK-432 (Picibanil; Chugai Pharmaceuticals, Tokyo, Japan), a penicillin-killed and lyophilized preparation of a low-virulence strain (Su) of *Streptococcus pyogenes* (group A) [21].

In vitro analysis of DC functions

Flow cytometric analysis, measurement of IL-12p70 production, T-cell–stimulatory capacity of DCs for allogeneic naive $CD4^+$ T cells, and the cytokine profile of $CD4^+$ T cells primed with DCs were analyzed as described previously [15,22].

Uptake of apoptotic cells by DCs and the cross-presenting capacity of DCs

Efficiency of uptake of apoptotic cells by DCs was assessed as described previously [23] using myeloid leukemia cell lines K562, OUN-1 [24] (Dr. Yasukawa, Ehime University, Japan), and a T-cell leukemia cell line MT2, which were killed by 120 Gy γ-irradiation and 48-hour serum-free culture in RPMI-1640 (Wako Pure Chemical Industries, Osaka, Japan). To examine the cross-presenting capacity of DCs, human leukocyte antigen (HLA)-A*2402–positive, immature 3d-DCs were pulsed with HLA-A*2402–negative, Epstein-Barr virus–transformed lymphoblastoid cell lines, which were killed as described here. DCs were matured with OK-432 (0.1 KE/mL) and prostaglandin E_2 (1 μg/mL) (MP Biomedicals, Solon, OH, USA) for 6 hours, and cocultured with autologous T cells at a DC-to-T cell ratio of 1:10. IL-2 (50 IU/mL; Teceleukin; Shionogi & Co., Ltd., Osaka, Japan) was added on the next day. For a positive control, DCs pulsed with HLA-A*2402–restricted EBNA3B peptide (TYSAGIVQI; KURABO Industries, Osaka, Japan) were used. Expansion of EBNA3A- and EBNA3B-specific $CD8^+$ T cells were evaluated by HLA tetramer staining [25].

Clinical trial protocol

The protocol was approved by the Ethics Committee, Graduate School and Faculty of Medicine, Kyoto University. Each patient gave written informed consent in accordance with the Declaration of Helsinki. The primary and secondary objectives were the assessment of safety and immunological and clinical responses, respectively.

Autologous leukemic cells were harvested before induction chemotherapy. Patients were required to be between 16 and 79 years of age and have a diagnosis of AML according to World Health Organization criteria [26,27]. Patients were excluded if they had another concurrent malignancy, an active autoimmune disease, positivity for blood-borne infectious agents, or a history of penicillin allergy (because OK-432 contains penicillin). Patients were enrolled if 5×10^7 or more leukemic cells were harvested. Thereafter, patients were treated with chemotherapy. More than 4 weeks after the last chemotherapy, patients proceeded to the DC vaccination if leukemic cells in bone marrow (BM) were $<20\%$. In addition, to assess the clinical efficacy of DC vaccination, the presence of an evaluable lesion in BM, which was defined as 0.1% or more of leukemic cells by flow cytometry, was required. Furthermore, patients should have an Eastern Cooperative Oncology Group performance status of 0 to 2 and adequate vital organ functions. Patients were excluded if they had eligibility for hematopoietic stem cell transplantation or an uncontrollable infection. Concomitant chemotherapy and radiotherapy were prohibited.

DC vaccine generation

DC vaccines were generated from autologous monocytes under current Good Manufacturing Practice conditions. Autologous leukemic cells to be used as antigens were obtained as mononuclear cells (MNCs) by density gradient centrifugation over Ficoll-Hypaque (GE Healthcare, Buckinghamshire, UK) from BM and/or peripheral blood (PB) samples. MNCs were frozen in CP-1 freezing medium and stored at −150°C. Before added to DCs, MNCs were killed by 120 Gy

γ-irradiation and 48 hours serum starvation. Killing of MNCs was confirmed by the percentage of Annexin V–positive cells being 90% or more by flow cytometry and reduced uptake of [^{3}H]-thymidine to the baseline level.

Apheresis products, which were obtained with COBE Spectra (Caridian BCT, Lakewood, CO, USA) from 10 L blood, were processed by elutriation using Elutra (Caridian BCT) to enrich monocytes. At the time of apheresis, no leukemic cells were observed in the PB of the patients, as assessed by a routine clinical laboratory test. Monocytes were cultured with 800 U/mL GM-CSF and 500 U/mL IL-4 in CellGro DC medium in gas-permeable plastic bags (VueLife 118; CellGenix Technologie Transfer) at 37°C, 5% CO_2 to generate immature DCs. After 48 hours, DCs were pulsed with autologous apoptotic leukemic cells and 2 μg/mL keyhole-limpet hemocyanin (KLH; Biosyn Corporation, Carlsbad, CA, USA). The endotoxin level in the KLH preparation examined by the supplier was <0.1 IU/mg. After an additional 24 hours, DCs were frozen as immature DCs in CP-1 freezing medium and stored at −150°C.

Administration of the DC vaccine

A total of 1×10^7 DCs were intradermally injected at four sites in bilateral arms and thighs. Twenty-four hours before DC administration, the injection sites were pretreated by 0.2 KE/site OK-432. At the time of DC administration, DCs were thawed and mixed with 1 KE OK-432. Then, the mixture of DCs and OK-432 was injected. The DC administration was repeated at 2-week intervals for five administrations.

Monitoring of immunological and clinical responses

Antigen-specific immune responses were assessed at indicated time points. Immune responses to KLH and autologous leukemic cells were tested by skin delayed-type hypersensitivity tests and interferon (IFN)-γ enzyme-linked immunospot (ELISPOT) assays. In addition, in a HLA-A*2402–positive patient, immune responses to HLA-A*2402–restricted peptides derived from leukemia-associated antigens were examined by IFN-γ ELISPOT assay and HLA tetramer staining. The peptides used in the assays were the natural $WT1_{235-243}$ peptide (CMTWNQMNL) [24], the modified $WT1_{235-243}$ peptide (CYTWNQMNL) [28], the human telomerase reverse transcriptase $(hTERT)_{461-469}$ peptide (VYGFVRACL) [29], and the lower matrix 65-kd phosphoprotein (pp65) of cytomegalovirus (CMV) (amino acids 328–336; QYDPVAALF) [30]. All peptides were purchased from Multiple Peptide Systems (San Diego, CA, USA). Both PBMCs and BM mononuclear cells (BMMCs) were subjected to assays before and after 1-week in vitro stimulation with antigen- or peptide-pulsed DCs in the presence of 15 U/mL IL-2 (Teceleukin). To evaluate clinical responses, percentages of leukemic cells in BM were monitored by morphology and flow cytometry at indicated time points.

Skin delayed-type hypersensitivity test

The 4×10^5 antigen-pulsed DCs were intradermally injected in the forearm. Sizes of induration and erythema were measured 48 hours later. Erythema that was 1.5-fold or larger in diameter than the antigen-unpulsed control was considered positive.

IFN-γ ELISPOT assay

IFN-γ ELISPOT assays (Mabtech, Nacka Strand, Sweden) were performed using antigen-pulsed DCs and peptide-pulsed C1R-A*2402 (Dr. Masafumi Takiguchi, Kumamoto University, Kumamoto, Japan). Stimulator cells were plated at 2×10^4 cells/well. As responder cells, fresh and in vitro–stimulated MNCs from PB and BM were plated with fresh MNCs at 1 to 2×10^5 cells/well and in vitro–stimulated MNCs at 1 to 2×10^4 cells/well. After overnight incubation, spots were developed using 3-amino-9-ethylcarbazole (Sigma Chemical, St Louis, MO, USA) and counted by KS ELISPOT compact (Carl Zeiss MicroImaging, Tokyo, Japan). Numbers of specific spot-forming cells were calculated by subtracting the number of spots with unpulsed DCs from the number of spots with antigen-pulsed DCs.

HLA tetramer staining

Natural $WT1_{235-243}$ peptide/HLA-A*2402 tetramer was purchased from Medical & Biological Laboratories (Nagoya, Japan). Modified $WT1_{235-243}$ peptide/HLA-A*2402 tetramer and a peptide derived from the HIV envelope (env) protein/HLA-A*2402 tetramer were produced as described previously [30]. Fresh and in vitro–stimulated MNCs were stained with a tetramer and fluorescein isothiocyanate–conjugated anti-CD8 monoclonal antibody (BD Biosciences) and analyzed by flow cytometery (FACSCalibur; BD Biosciences) [30].

Results

In vitro assays to optimize generation of DCs

To optimize generation of DCs, we performed in vitro functional assays. We first compared DCs differentiated from monocytes in the presence of GM-CSF and IL-4 for 3 days with 6-day differentiated DCs conventionally used in clinical trials. After 24-hour exposure to OK-432, both 3d-DCs and 6d-DCs showed similar levels of surface molecule expressions, IL-12p70 production, and T-cell stimulatory capacity for allogeneic naïve $CD4^+$ T cells (Supplementary Figure E1; online only, available at www.exphem.org), indicating that 3d-DCs have functions comparable with 6d-DCs. Next, we examined the capacity of 3d-DCs to cross-present apoptotic cell–associated antigens. At the DC-to-apoptotic cell ratio of 1:1, 11% to 33% of immature 3d-DCs incorporated apoptotic leukemia cell lines (Fig. 1A). Moreover, HLA-A*2402–positive DCs pulsed with killed lymphoblastoid cell lines from an HLA-A*2402–negative donor induced expansion of $CD8^+$ T cells specific for the HLA-A*2402–restricted epitopes of EBNA3A and EBNA3B (Fig. 1B), indicating the capacity of DCs to cross-present apoptotic cell–derived antigens.

An extended period of exposure of DCs to lipopolysaccharide leads to DC exhaustion [17], as indicated by loss of IL-12–producing capacity by DCs. To examine whether OK-432 induces DC exhaustion, we analyzed the maturation kinetics of OK-432–stimulated 3d-DCs. Upregulation of the surface molecules (Fig. 2A) and IL-12p70 production (Fig. 2B) became evident 4 and 8 hours after OK-432 stimulation, respectively. Maximal levels of surface molecule expressions and IL-12p70 production were observed at 48 hours. Next, we examined how many hours of exposure to OK-432 is sufficient to elicit a maturation signal to DCs, using 3d-DCs that were cultured for a total of 48 hours with different

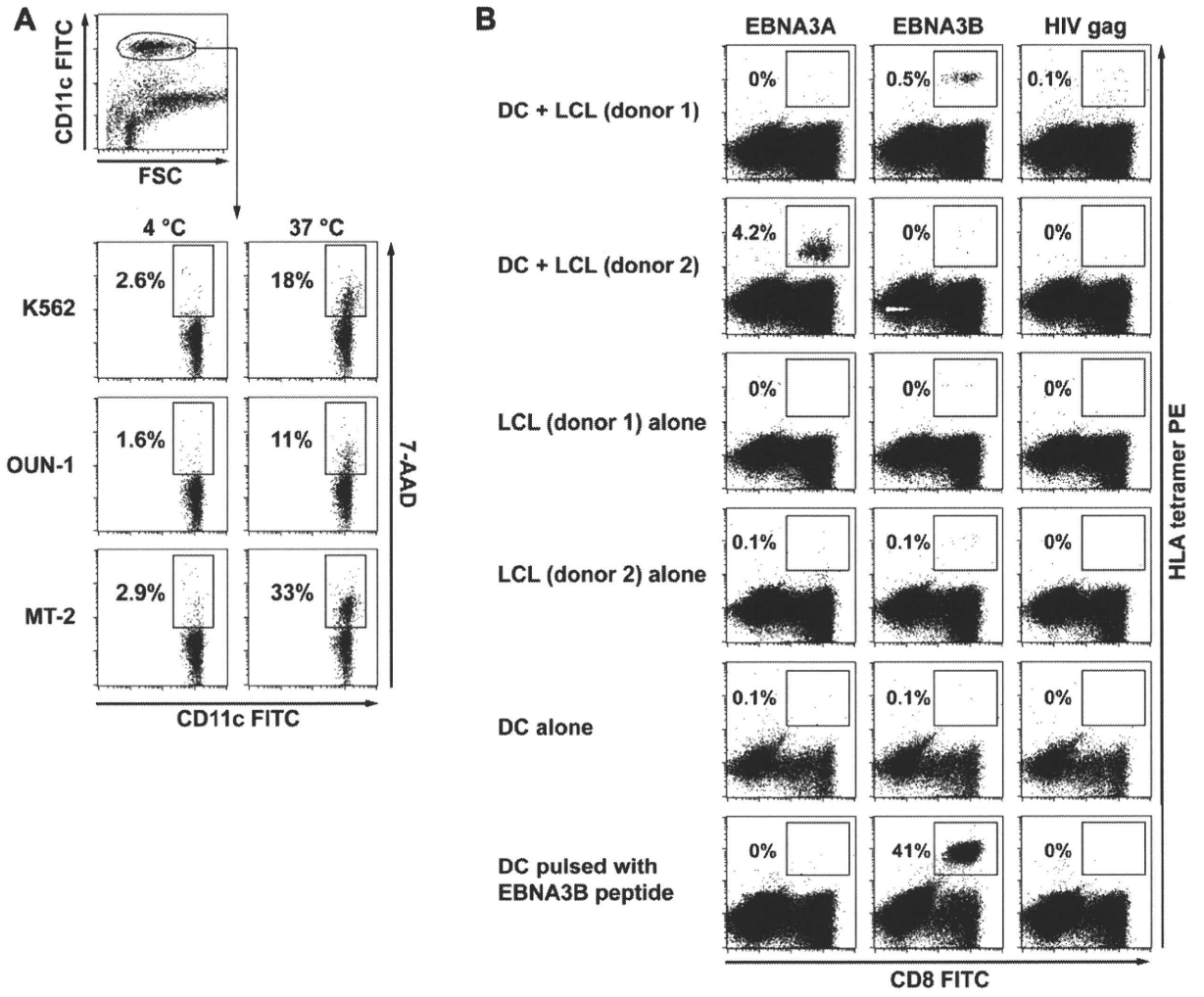

Figure 1. 3d-DCs incorporate apoptotic cells and cross-present cell-associated antigens. (**A**) Uptake of apoptotic cells by 3d-DCs. Apoptotic K562, OUN-1, and MT2 were labeled with 7-aminoactinomycin D (7-AAD) (20 μg/mL), and cocultured with immature 3d-DCs at a DC-to-apoptotic cell ratio of 1:1. After 4 hours of incubation at 4°C or 37°C, cells were stained with fluorescein isothiocyanate–conjugated anti-CD11c monoclonal antibody and analyzed by flow cytometry. Cells positive for both CD11c and 7-AAD were considered to be DCs that had phagocytosed apoptotic cells. (**B**) The cross-presenting capacity of DCs. Immature 3d-DCs from a HLA-A*2402–positive donor were pulsed with apoptotic HLA-A*2402–negative donor-derived lymphoblastoid cell lines (LCLs), matured with OK-432 and prostaglandin E_2, and cocultured with autologous T cells. For a positive control, DCs pulsed with the EBNA3B peptide were used as a stimulator. After 7 days, expansions of EBNA3A- and EBNA3B-specific $CD8^+$ T cells were evaluated by HLA tetramer staining. Dead cells are excluded by staining with propidium iodide. Numbers shown indicate percentages of tetramer-positive cells among $CD8^+$ cells. Representative data from two experiments are shown.

durations of exposure to OK-432 at the start of culture. As short as 2-hour exposure upregulated CD83 and CD86 (Fig. 2C) and induced IL-12p70 production (Fig. 2D) during the subsequent 46-hour culture without OK-432. Although at the time of 8-hour exposure, the induction of CD83, CD86 (Fig. 2A), and IL-12p70 (Fig. 2B) was low, 8-hour exposure was sufficient to induce maximal levels of CD83 and CD86 expression (Fig. 2C) and IL-12p70 production (Fig. 2D). Notably, although initial 24-hour exposure to OK-432 induced the maximal levels of CD83 and CD86 expression (Fig. 2C), DCs did not produce a detectable level of IL-12p70 during the last 24-hour culture (Fig. 2D). These data indicate that, like lipopolysaccharide [17], OK-432–induced IL-12p70 production was limited within the first 24 hours and most active between 8 and 24 hours after OK-432 stimulation. The functional significance of ongoing IL-12p70 production by DCs in priming naïve $CD4^+$ T cells was supported by the data that 3d-DCs matured with OK-432 for 6 hours showed a superior capacity to induce IFN-γ–producing T cells to those matured for 24 hours (Fig. 2E). Thus, extended stimulation with OK-432 induces DC exhaustion. To avoid it, we decided to administer immature DCs together with OK-432 to patients and to induce DC maturation in vivo.

It is convenient to prepare a large number of DCs from a single batch of apheresis and freeze them in aliquots. We assessed the effect of cryopreservation on DCs. Whereas cryopreserved immature 3d-DCs showed somewhat higher percentages of dead cells after 24-hour culture with or

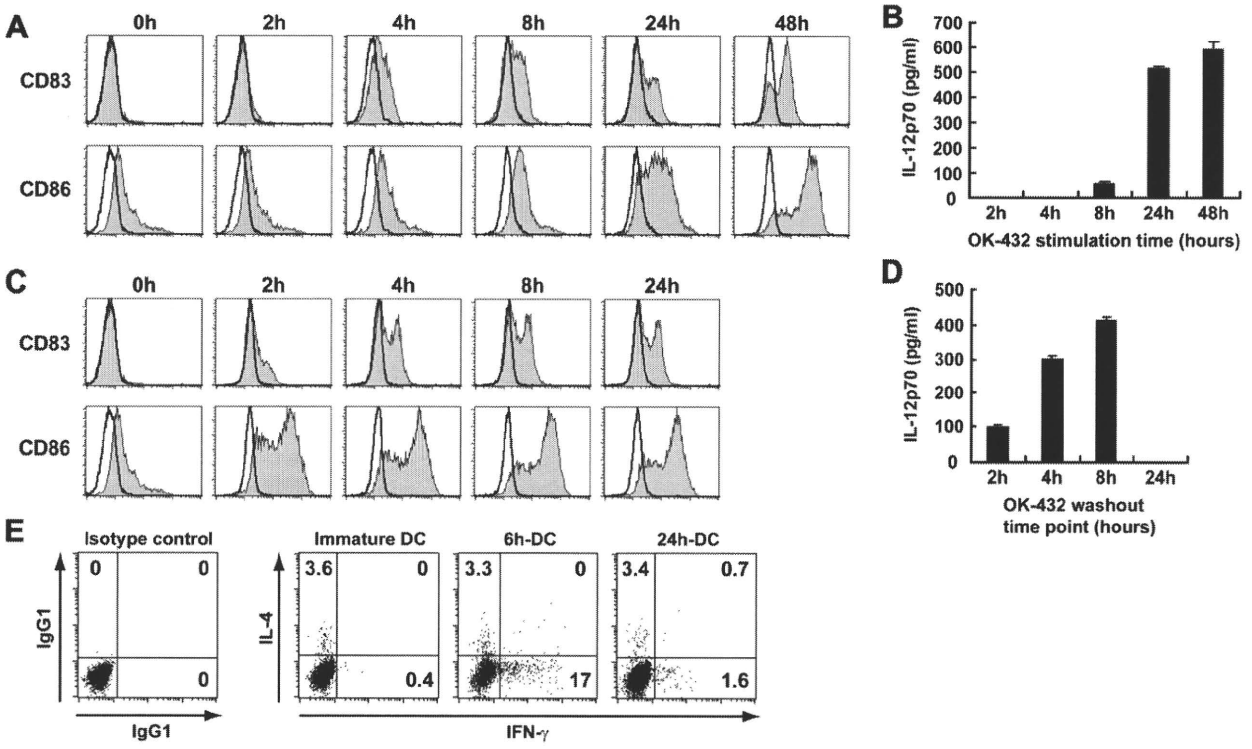

Figure 2. Short-term stimulation with OK-432 is optimal to generate Th1-inducing mature DCs. (**A**, **B**) Immature 3d-DCs were cultured in the presence of OK-432 (0.1 KE/mL) for indicated time periods, then harvested and analyzed. (**C**, **D**) Immature 3d-DCs were cultured in the presence of OK-432 for indicated time periods, washed, replated, and further cultured for a total of 48 hours. Cells and supernatants harvested at 48 hours were analyzed. (**A**, **C**) Expression of CD83 and CD86 was analyzed by flow cytometry. Dead cells were excluded by staining with propidium iodide. Open histograms indicate staining with isotype controls. (**B**, **D**) IL-12p70 production in culture supernatants of DCs (5×10^5 cells/mL) were measured by enzyme-linked immunosorbent assay. Error bars indicate the standard deviation of duplicate measurements. (**E**) Naïve $CD4^+$ T cell differentiation induced by DCs. Immature 3d-DCs were matured with OK-432 (0.1 KE/mL) for 6 or 24 hours and cocultured with allogeneic naïve $CD4^+$ T cells for 7 days. Cytokine profiles of T cells were analyzed by intracellular cytokine staining. Numbers indicate percentages of cells in each quadrant. Representative data from four experiments are shown.

without OK-432, and tended to produce a lower amount of IL-12p70 upon OK-432 stimulation as compared with non-cryopreserved DCs (Supplementary Figure E2A, C; online only, available at www.exphem.org), similar levels of CD83 and CD86 expression were induced by OK-432 in both DCs (Supplementary Figure E2B; online only, available at www.exphem.org). Thus, although cryopreservation of immature DCs impaired their function to some extent, cryopreserved DCs largely retained the viability and expression of immunostimulatory molecules. Considering the practical convenience to prepare a stock of DCs at one time, we decided to freeze DCs as immature DCs. Taken together, these data demonstrate that DCs generated in the present study are capable of inducing $CD8^+$ T-cell responses to apoptotic cell–derived antigens, and that immature DCs can be cryopreserved without critical loss of functions.

Patients, feasibility, and safety

Thirteen patients were recruited to the study for the leukemic-cell harvest at the onset of AML. After chemotherapy, four patients were eligible for DC vaccination (Table 1). In these patients, $>5 \times 10^7$ DCs for five vaccinations could be generated from a single apheresis. Autologous apoptotic leukemic cells were added to DCs as antigens at leukemic cell-to-DC ratios of 1:3.3 to 1:6.5, depending on the numbers of collected leukemic cells (Supplementary Table E1; online only, available at www.exphem.org). Status of PB and BM at the time of apheresis are shown in Supplementary Table E1 (online only, available at www.exphem.org). Representative data of surface molecule expressions on DCs are shown in Supplementary Figure E3 (online only, available at www.exphem.org).

All of the patients completed the five vaccinations safely (Table 1). In all the patients, grade 1 to 2 fever and grade 2 skin reactions at the injection sites were observed. The fever was resolved within 2 days after vaccination and most likely related to administration of OK-432. The skin reactions at the injection sites were transient and characterized by erythema, pruritus, and tenderness. No significant toxicities to vital organs or signs of autoimmunity were observed.

Induction of antigen-specific immune responses to KLH and leukemic cells

Induction of an immune response to KLH was detected by skin delayed-type hypersensitivity tests and/or IFN-γ ELISPOT assays in three patients, with the exception of patient no. 4

Table 1. Patient characteristics and results of the DC vaccination

Patient no.	Age/Sex	Diagnosis	DC vaccination was started		LC in BM at the first vaccination[a] (%)	Adverse effects[b]	Immune response		Clinical response	Died at (days after the last vaccination)
			After the last CT (d)	After diagnosis (d)			KLH	LC		
1	76/F	AML-MRC	82	93	1.8	Fever (1) Injection site reaction (2)	Yes	No	PD Died of sepsis with leukemia	186
2	75/M	AML-MRC	40	155	0.6	Fever (1) Injection site reaction (2)	Yes	Yes	Transient disease stabilization Died of leukemia	391
3	70/M	AML-MRC	44	344	2.9	Fever (2) Injection site reaction (2)	Yes	Yes	Transient disease stabilization Died of sepsis with leukemia	192
4	66/M	AML M2	67	144	0.2	Fever (1) Injection site reaction (2)	No	No	PD Died of leukemia	66

AML-MRC = acute myeloid leukemia with myelodysplasia-related changes; CT = chemotherapy; F = female; LC = leukemic cells; M = male; PD = progressive disease.
[a]Percentages of leukemic cells in bone marrow were determined by flow cytometry.
[b]Numbers in parentheses indicate grade of toxicity according to the National Cancer Institute-Common Terminology Criteria for Adverse Events version 3.0.

(Table 1 and data not shown). Two patients (patient nos. 2 and 3) showed induction of immune responses to leukemia-associated antigens. In patient no. 2, who was HLA-A*2402–negative, IFN-γ ELISPOT assays using autologous leukemic cell–pulsed DCs revealed the induction of antileukemic immunity in PBMCs and BMMCs without in vitro stimulation after the fourth vaccination (Fig. 3A). The antileukemic immune response was still detected 1 month after the fifth vaccination in in vitro–stimulated PBMCs and BMMCs (Fig. 3B), but was no longer detected without in vitro stimulation (Fig. 3A). We could not test antileukemic immunity at subsequent time points in this patient because the patient developed leukocytopenia, probably owing to progression of myelodysplastic syndrome.

In patient no. 3, who was HLA-A*2402–positive, HLA-A*2402–restricted peptides from WT1 and hTERT were used in immunological monitoring. $CMVpp65_{328–336}$ peptide was used as a positive control in ELISPOT assays (Fig. 4B). No responses to the leukemia-associated antigens were observed until the fourth vaccination. However, 2 months after the fifth vaccination, positive responses to the modified $WT1_{235–243}$ and the $hTERT_{461–469}$ peptides were detected in in vitro–stimulated PBMCs by HLA tetramer staining (Fig. 4A) and an IFN-γ ELISPOT assay (Fig. 4B), respectively. The PBMCs binding to the modified $WT1_{235–243}$ peptide/HLA-A*2402 tetramer also bound to the natural $WT1_{235–243}$ peptide/HLA-A*2402 tetramer (Fig. 4A), indicating that these cells were capable of recognizing the natural WT1 peptide presented on leukemic cells. These responses were short-lived and almost completely disappeared 3 months after the fifth vaccination. No responses were detected in PBMCs or BMMCs without in vitro stimulation (data not shown). Thus, the vaccinations induced HLA class I–restricted, antileukemic immunity, indicating that the DCs cross-presented leukemia-associated antigens in vivo. In addition, in patient no. 2, leukemic cell-reactive T cells were detected in BM (Fig. 3), the main tumor site in leukemia.

Clinical outcomes

The two patients with antileukemic immunity had longer periods of disease stabilization than the other two patients without antileukemic immunity (Fig. 5A). Notably, in patient no. 3, the percentages of leukemic cells in BM dropped from 11% to 5.2% during the second month after the fifth vaccination, when a positive antileukemic immunity was observed (Fig. 5B). Thus, these observations suggest that induction of antileukemic immunity was associated with extended the periods of disease stabilization in these patients.

Discussion

Novel therapies with less toxicity are necessary for intractable AML in elderly patients. In this study, we conducted a phase I clinical trial of immunotherapy for such patients using DCs pulsed with autologous apoptotic leukemic cells.

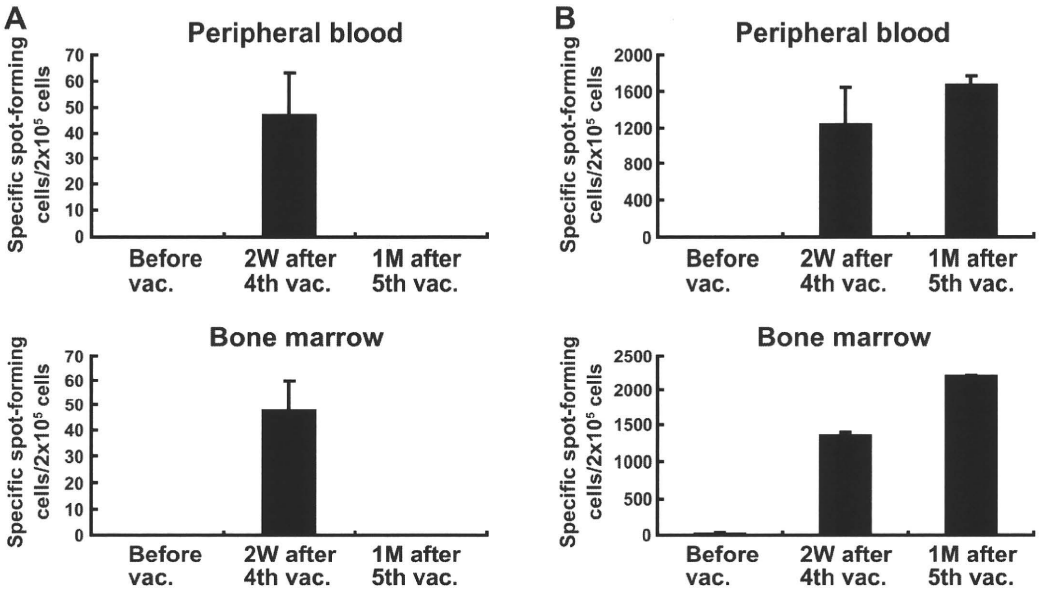

Figure 3. IFN-γ ELISPOT assay in patient no. 2. MNCs from PB and BM were obtained at indicated time points and subjected to IFN-γ ELISPOT assays directly after isolation (**A**) or after 1 week of stimulation with antigen-pulsed DCs (**B**). In IFN-γ ELISPOT assays, 2×10^5 MNCs (**A**) and 1×10^4 MNCs (**B**) were incubated with 1×10^4 leukemic cell–pulsed or unpulsed DCs. Numbers of specific spot-forming cells per 2×10^5 MNCs, calculated by subtracting numbers of spots with unpulsed DCs from numbers of spots with leukemic cell–pulsed DCs. Error bars indicate the standard deviation of duplicate measurements.

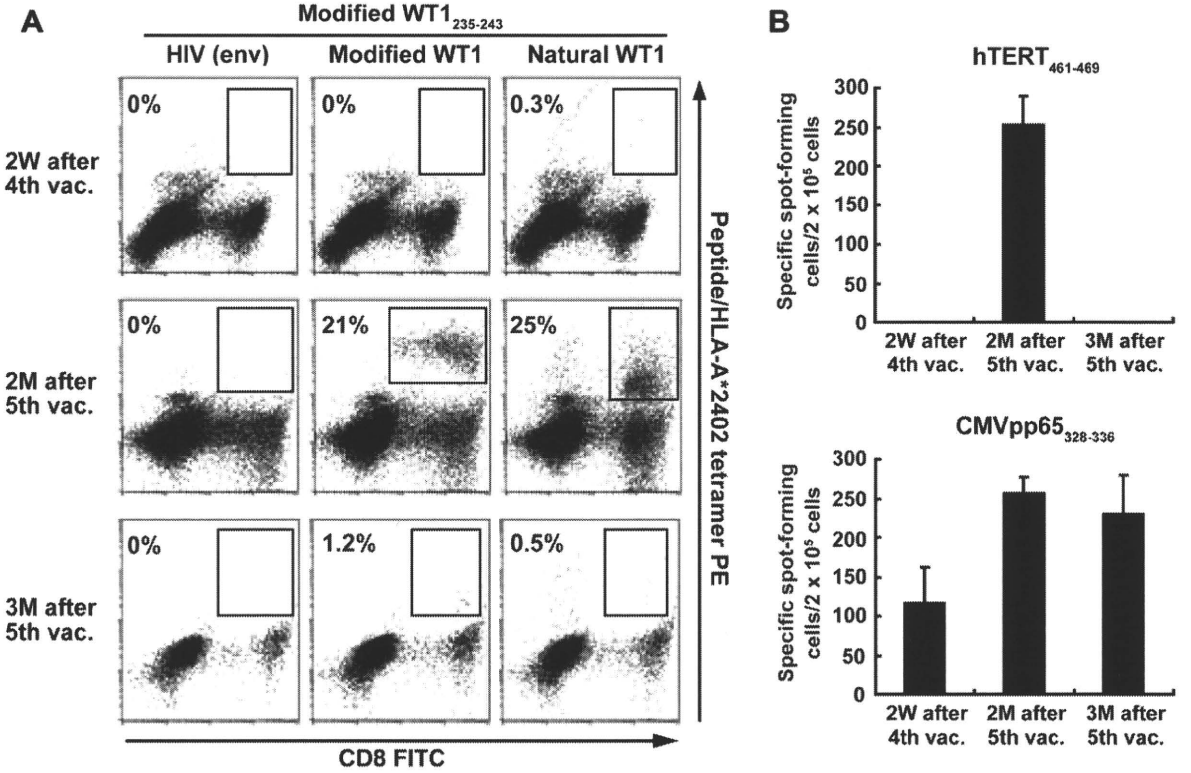

Figure 4. Immune responses in patient no. 3. (**A**) HLA tetramer staining. MNCs from PB were obtained at indicated time points, stimulated for 1 week with DCs pulsed with the modified $WT1_{235-243}$ peptide, stained with phycoerythrin-labeled peptide/HLA-A*2402 tetramers and fluorescein isothiocyanate–labeled anti-CD8 monoclonal antibody, and analyzed by flow cytometry. Dead cells were excluded by staining with propidium iodide. Numbers indicate percentages of tetramer-positive cells among $CD8^+$ cells. (**B**) IFN-γ ELISPOT assay. MNCs were stimulated for 1 week with DCs pulsed with the $hTERT_{461-469}$ or $CMVpp65_{328-336}$ peptide, and subjected to IFN-γ ELISPOT assays. In the assays, 2×10^4 MNCs were incubated with 2×10^4 C1R-A*2402 pulsed with or without the $hTERT_{461-469}$ or $CMVpp65_{328-336}$ peptide. Before vaccination, the assay was performed using DCs as a stimulator, which induced many nonspecific spots. Thus, the data before vaccination are not shown. Numbers of specific spot-forming cells per 2×10^5 MNCs, calculated by subtracting numbers of spots with unpulsed C1R-A*2402 from numbers of spots with antigen-pulsed C1R-A*2402, were depicted. Error bars indicate the standard deviation of duplicate measurements.

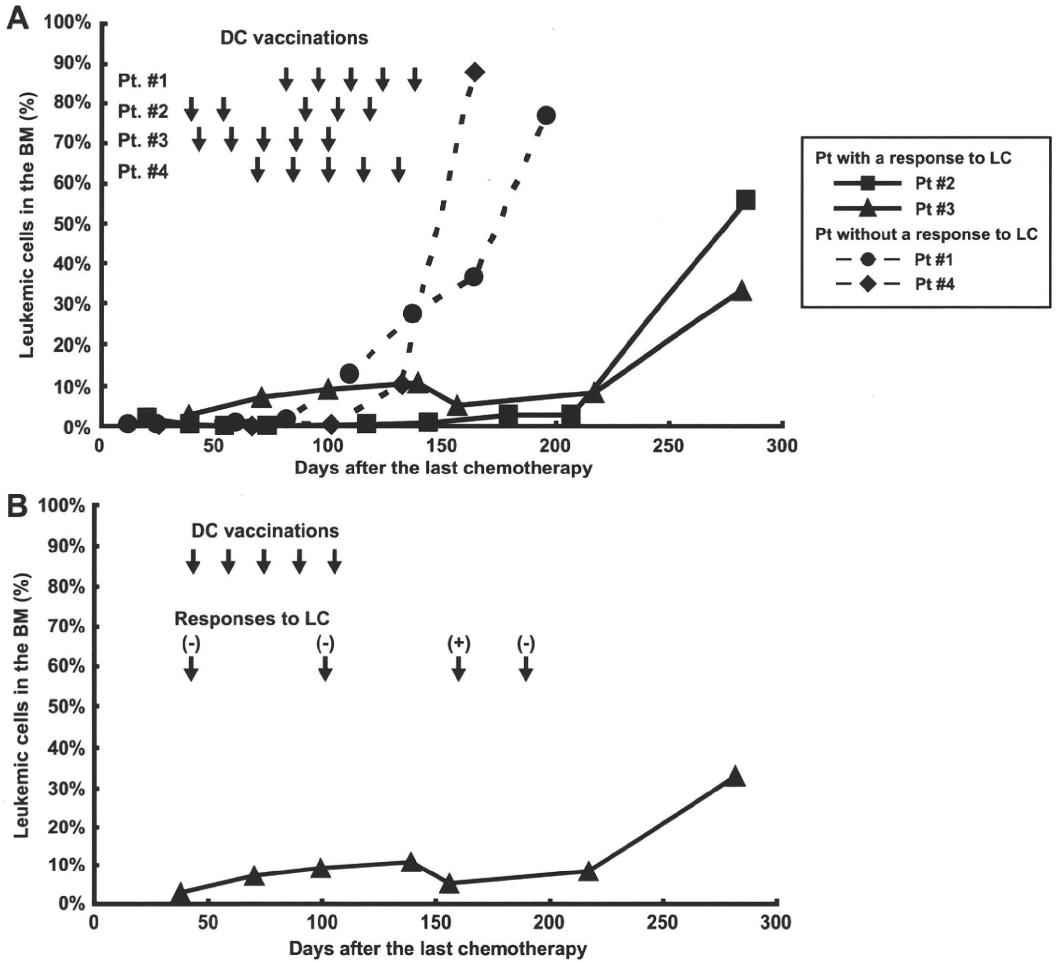

Figure 5. Clinical courses during the DC vaccination. (**A**) Percentages of leukemic cells in BM as determined by flow cytometry in four vaccinated patients are shown. Solid lines indicate patients with immune responses to leukemic cells (LCs) (patients 2 [■] and 3 [▲]). Dashed lines indicate patients without immune responses to LCs (patients 1 [●] and 4 [◆]). Arrows indicate time points when DC vaccines were administered to each patient. (**B**) The clinical course of patient no. 3. Arrows indicate time points when immunological monitoring was performed. Plus (+) or minus (−) signs indicates that immune responses to leukemic cells were detected or not detected at that time point, respectively.

Induction of antileukemic immunity was observed in two of four vaccinated patients. This is the first study that demonstrates cross-priming of $CD8^+$ T cells by DCs pulsed with apoptotic leukemic cells in vivo in humans, thus providing a proof of principle of this approach. The limited number of patients prevented us from drawing any definitive conclusion regarding clinical efficacy from the present trial. However, longer periods of disease stabilization observed in the two patients with antileukemic immunity compared to the other two patients without antileukemic immunity implied that induction of antileukemic immunity might have impacted on the clinical course of these patients.

There are several features in the method of DC vaccination in this trial: short-term 3-day culture to generate DCs in an attempt to reduce labor, cost, and time; use of whole leukemic cells as antigens to induce multivalent immune responses; use of the microbial adjuvant OK-432 as a maturation-inducing factor to generate Th1-inducing DCs; in vivo maturation of DCs to avoid DC exhaustion by extended stimulation in vitro with OK-432; and prior induction of inflammation at the injection sites to facilitate DC migration to draining lymph nodes.

We used autologous apoptotic leukemic cells as antigens because several studies have shown that apoptotic cells are more efficiently cross-presented by DCs to $CD8^+$ T cells than soluble antigens such as tumor lysate [31–34]. Furthermore, MoDCs has been shown to cross-present apoptotic leukemic cells to $CD8^+$ T cells in vitro [35]. Apoptotic cells as antigens also have advantages over peptides, in that the DCs have the ability to process multiple antigens from the apoptotic cells and present those antigens on their own HLA molecules. In this study, we clearly showed that MoDCs cross-presented leukemia-associated antigens WT1 and hTERT from apoptotic leukemic cells. Furthermore, T cells reactive to leukemic cells were detected in BM.

A murine study has shown that DC maturation not by inflammatory cytokines but by pathogen-derived components is crucial for DCs to acquire the capacity to differentiate naïve $CD4^+$ T cells into effector T cells [16]. We used OK-432, a preparation of killed *Streptococcus pyogenes* [21], which strongly triggers DC maturation through Toll-like receptor 4 [36–39]. We showed that, like lipopolysaccharide [17], longer stimulation with OK-432 induces DC exhaustion, resulting in the reduced capacity of DCs to induce Th1 responses. Several preclinical studies have shown that DCs briefly exposed to Toll-like receptor ligands are better inducers of Th1-type and cytotoxic T-cell responses [17,40,41]. Moreover, a clinical trial suggests superiority of briefly matured DCs in pediatric patients with cancer [42]. In this trial, we administered immature DCs together with OK-432 to avoid DC exhaustion before administration. The induction of IFN-γ detected by the ELISPOT assay implied IL-12 production by DCs in vivo.

Only a small proportion of intradermally administered DCs reach draining lymph nodes [43,44]. In a mouse model, pretreatment of administration sites with inflammatory cytokines enhance DC migration to regional lymph nodes [18]. Based on this finding, we pretreated administration sites with a low dose of OK-432. Because of unavailability of a cell-processing facility for cells labeled with indium-111 oxyquinoline [43,44], we could not evaluate the efficiency of DC migration to lymph nodes. Whether this administration procedure is superior to others should be evaluated in future studies.

In this study, multiple vaccinations were required to elicit antileukemic immunity, which rapidly declined after cessation of vaccination. Maintenance of antileukemic immunity might lead to improvement of clinical efficacy, and might be fulfilled by increasing the number of vaccinations, which was, however, impossible in this study because of the limited availability of autologous leukemic cells. Thus, if a peptide is available for the induced antileukemic $CD8^+$ T-cell response, peptide vaccination may be added after DC vaccination. Furthermore, blockade of immunosuppressive mechanisms may be combined.

In conclusion, we demonstrated the feasibility, safety, and immunogenicity of DC-based immunotherapy for elderly patients with AML. Cross-priming of $CD8^+$ T cells by DCs pulsed with autologous apoptotic leukemic cells was provoked in vivo. The results were promising, yet further intensification of vaccine potency is clearly required. This novel therapeutic approach may lead to improvement of clinical outcomes in elderly patients with AML, which has been difficult to achieve with other therapeutic approaches.

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Conflict of interest disclosure

No financial interest/relationships with financial interest relating to the topic of this article have been declared.

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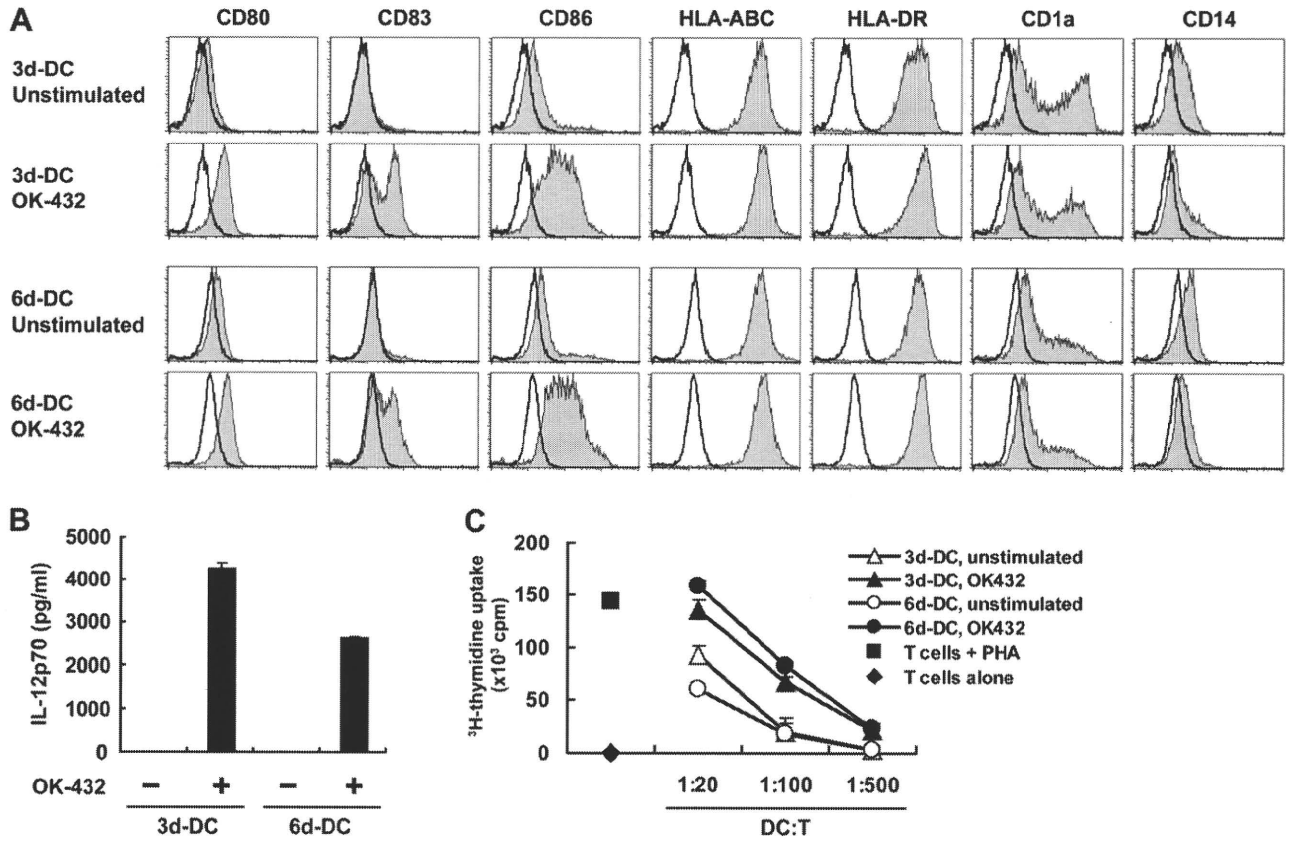
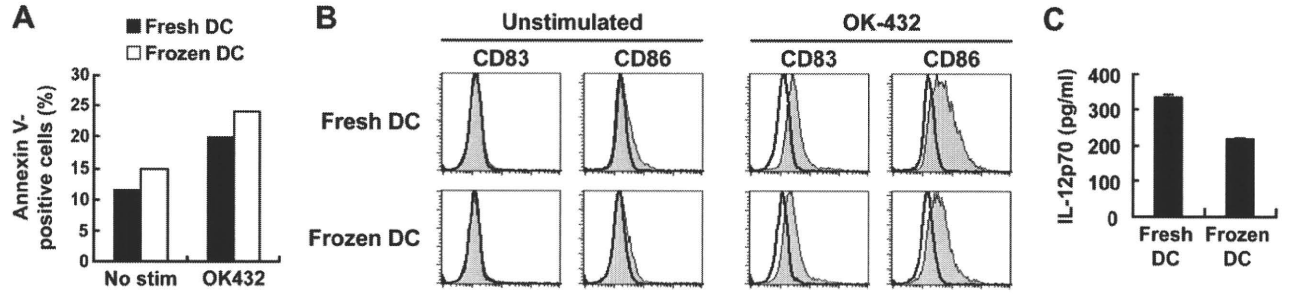


Supplementary Figure E1. 3d-DCs and 6d-DCs have comparable T-cell stimulatory capacity. (**A**) Expressions of surface molecules on DCs. Unstimulated or OK-432-stimulated DCs were analyzed by flow cytometry. Dead cells were excluded by staining with propidium iodide. Open histograms indicate staining with isotype controls. (**B**) IL-12p70 production by DCs (5×10^5 cells/mL) stimulated with OK-432 (0.1 KE/mL) for 24 hours was measured by enzyme-linked immunosorbent assay. Error bars indicate the standard deviation of duplicate measurements. (**C**) Proliferation of naive $CD4^+$ T cells stimulated with DCs. Allogeneic naive $CD4^+$ T cells were cocultured with DCs at indicated DC to T-cell ratios. On day 4, 1 Ci of [^{3}H]-thymidine was added. After 16 hours of further incubation, thymidine uptake was counted. Naive $CD4^+$ T cells were stimulated with 10 μg/mL phytohemagglutinin as a positive control. Representative data from three experiments are shown.



Supplementary Figure E2. Effects of cryopreservation on immature 3d-DCs. (**A**) Viability of fresh and frozen 3d-DCs after 24 hours of incubation with or without OK-432 (0.1 KE/mL) were evaluated by staining with Annexin-V. Percentages of Annexin-Vpositive cells are indicated. (**B**) Expression of surface molecules on fresh and frozen DCs after 24 hours of incubation with or without OK-432. (**C**) IL-12p70 production by fresh and frozen DCs (5×10^5 cells/mL) induced by 24-hour stimulation with OK-432 was measured by enzyme-linked immunosorbent assay. Error bars indicate the standard deviation of duplicate measurements. Representative data from four experiments are shown.