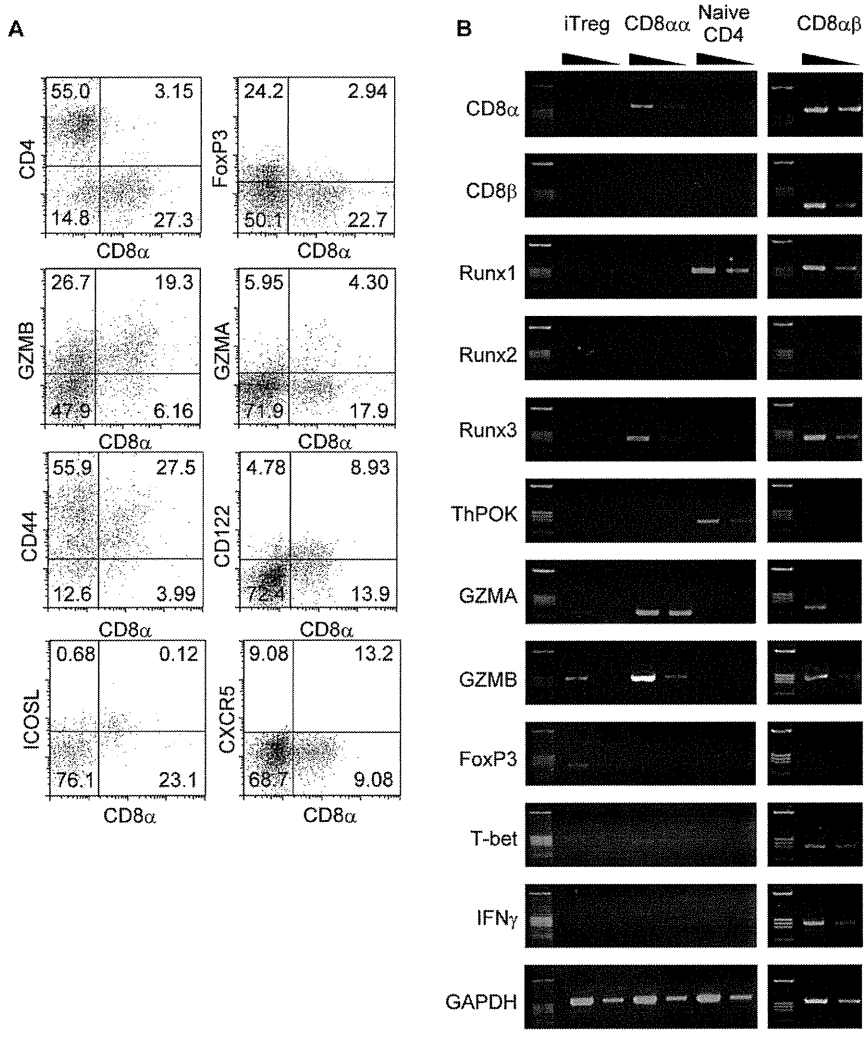

Figure 3 | Gene expressions of CD8ααTCRαβ Tregs differentiated *in vitro*. (A) Naïve CD4+ T cells were stimulated under conditions mentioned in Fig. 2(A). Gene expression was estimated by FACS. (B) Naïve CD4+ T cells were stimulated under conditions mentioned in Fig. 2(A). iTreg (CD4+T) and CD8αα T (CD8+ T) cells were sorted from the cultured cells (shown in Fig 3A), and gene expression was analyzed by reverse transcription–polymerase chain reaction. Naïve CD4 T cells (CD4+ CD62L+ T cells), CD8αβ T cells were used for control. Naive CD4, CD8αβ, iTreg, and CD8αα indicate RNA source used for cDNA generation. Fivefold serial dilutions of cDNAs were amplified for the transcripts indicated. All experiments were performed at least thrice, and representative data are shown.

mice were adoptively transferred into Rag2$^{-/-}$ mice with a C57BL/6 background (CD45.1), and the generation of CD8αα T cells was examined after atRA administration. Given that the homing location of transferred CD4 T cells cannot be controlled, absolute cell numbers of transferred T cells at PP, IEL, and LPL varied substantially. Accordingly, CD8αα T cell differentiation was examined by assessing the percentages of CD8αα cells. CD8αα T cells appeared in an atRA-dependent manner in all tissues examined (Fig. 5). The percentage of CD8αα T cells was significantly higher in PPs and IELs than that in controls (12.2% of CD8αα+CD4−TCRαβ+ T cells in atRA-treated PPs compared with 0.05% in controls; 10.0% in the atRA-treated sIEL compared with 0.09% in controls; Fig. 5). These results indicate that the atRA-dependent differentiation of CD4 T cells into CD8αα T cells occurred *in vivo*. To exclude the possibility that CD8αα T cells were derived from contaminated CD8αβ T cells, we assessed the influence of CD8αβ T cells on the generation of

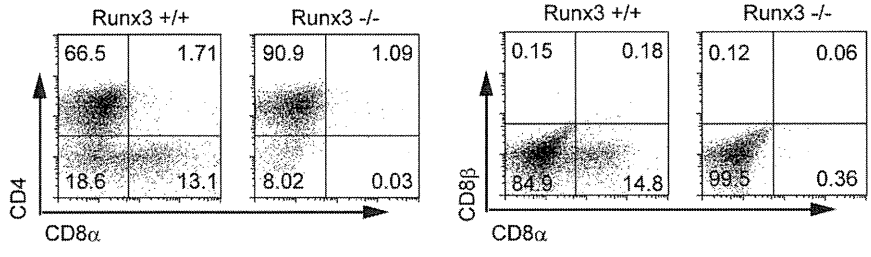

Figure 4 | Differentiation of CD8αα Tregs from CD4 T cells requires Runx3. Naïve CD4 T cells from wild type (WT) or Runx3$^{-/-}$ FL-derived cells were stimulated under the conditions mentioned in Fig. 2(A). CD8αα+CD4− T cells were analyzed by FACS. All experiments were performed thrice; representative data are shown.

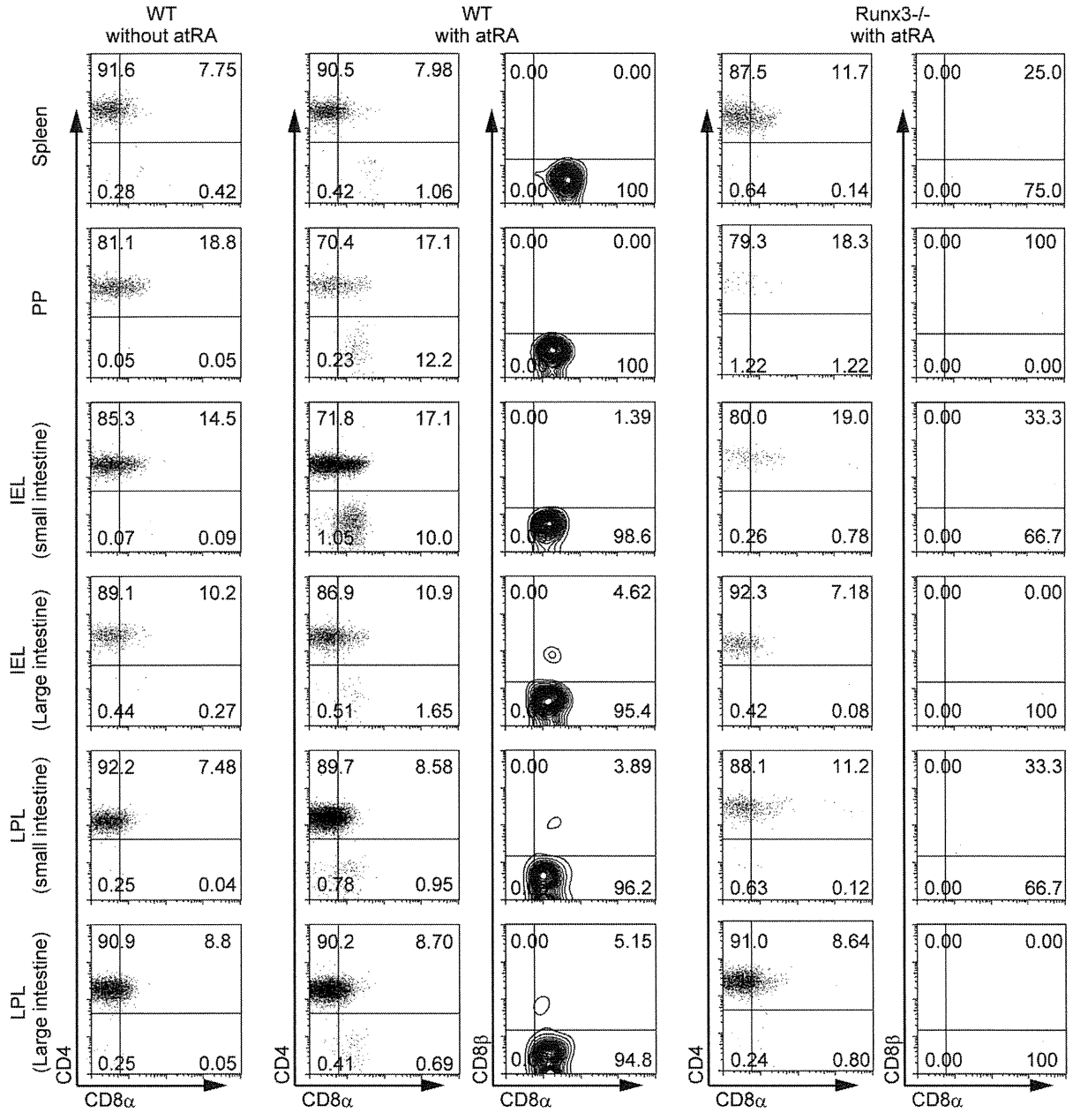

Figure 5 | Generation of CD8αα Tregs from CD4 T cells *in vivo* requires Runx3. FACS analysis of lymphocytes from Rag2$^{-/-}$ mice (CD45.1) that received naïve CD4 T cells (CD45.2) from WT or Runx3$^{-/-}$ FL-derived cells after atRA administration. TCRαβ+CD45.2+ cells were examined for CD4 and CD8α expression (all samples). CD4-CD8α+ T cells were further examined for CD8α and CD8β expression in case of atRA-administrated samples. All experiments were performed at least thrice; representative data are shown. LPLs, lamina propria lymphocytes.

CD8αα T cells by modulating the ratio of CD8αβ T cells within naïve CD4 T donor cells. Naïve CD4 T cells used for a series of cell transfer experiments contained fewer than 0.02% CD8αβ T cells. Purified naïve CD4 T cells were transferred into Rag2$^{-/-}$ mice with or without CD8αβ T cells, and the generation of CD8αα T cells *in vivo* was assessed. There was negligible difference in the percentage of CD8αα T cells between mice carrying CD4 T cell populations containing 0.02% CD8αβ T cells and those carrying populations containing 0.12% CD8αβ T cells (data not shown). In addition, naïve CD4 T cells, contaminated less than 0.002% by CD8αβ T cells, differentiated as efficiently as CD4 T cell populations carrying 0.02% CD8αβ T cells in Rag2$^{-/-}$ mice. These results indicate that CD8αβ T cells are not the major source of CD8αα T cells differentiated *in vivo*.

The contribution of Runx3 to differentiation of CD4-CD8αα *in vivo* was further determined using Runx3-deficient naïve CD4 T cells as donor. No CD8αα T cells were observed in either atRA-treated or non treated mice (Fig. 5). However, the preferential differentiation of CD8αβ+ T cells, but not CD8αα+ T cells, was observed following treatment with atRA in high doses (Supplementary Fig. S1). Considering all results, Runx3 plays a special role in the differentiation of CD8αα T cells from CD4 T cells *in vivo*. In addition, several other signals that were not examined in this study contribute to CD8 T cell generation from CD4 T cells *in vivo*; Runx3$^{-/-}$ naïve CD4 T cells cannot differentiate CD8α+ T cells *in vitro*. In view of the functional redundancy of Runx family transcription factors, their contribution to the differentiation of CD4 T cells into CD8 T cells was investigated using Runx2$^{-/-}$Runx3$^{-/-}$CD4 T cells. Administration of high-dose atRA could not rescue the defect in the generation of CD8 T cells (data not shown), indicating that Runx2 and Runx3 play critical roles in the differentiation of CD4 T cells into

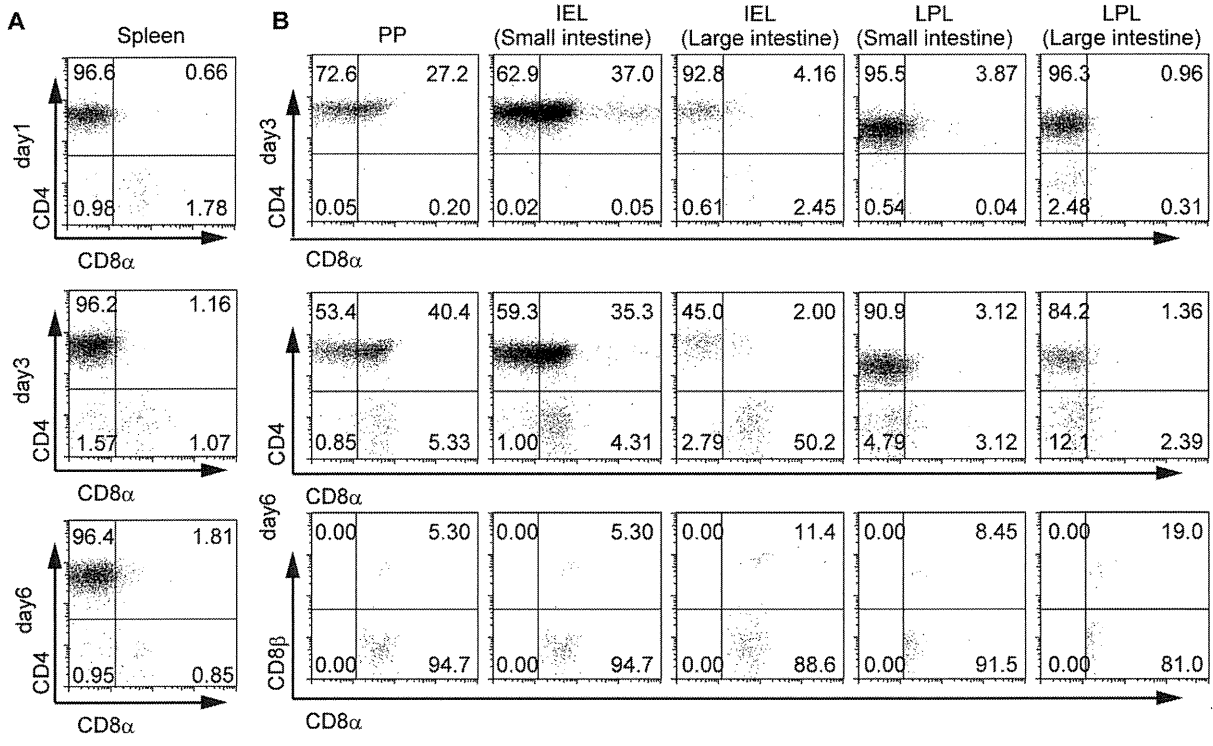

Figure 6 | CD4 T cells differentiate into CD8αα Tregs during immune responses. (A) Naïve CD4+ T cells (CD45.2) were injected into Rag2$^{-/-}$mice. TCRαβ+CD45.2+ cells from spleen at 1, 3 and 6 days after immunization were analysed for expression of CD8α and CD4. (B) FACS analysis of lymphocytes from tissues of mice used for (A). TCRαβ+CD45.2+ cells were examined for expression of CD4 and CD8α. CD4-CD8α+ T cells were further examined for expression of CD8α and CD8β. All experiments were performed at least three times, and representative data are shown.

CD8 T cells. However, the precise roles of each Runx factor in the differentiation into various CD4-derived CD8 T cell subsets remain to be investigated.

CD4 T cells differentiate into CD8αα Tregs during immune response and attenuate immune reactions. Accumulating evidence indicates that the gut is rich in atRA[28,29,36,37], and RA-dependent events are believed to occur preferentially in the gut. However, the spontaneous differentiation of CD4 T cells into CD8αα T cells was not observed in the gut in the absence of atRA administration (Fig. 5). The physiological relevance of differentiation of CD4 T cells into CD8αα T cells in normal immunological reactions is thus debatable. Recent reports have demonstrated that RA is required to promote the effector responses of CD4 T cells and is more abundant in inflamed tissues than in the gut[38,39]. We accordingly investigated whether the differentiation of CD4 T cells into CD8αα T cells can be induced by immunization. Generation of CD8αα T cells in Rag2$^{-/-}$ mice that received naïve CD4 T cells was assessed after immunization. On days 1, 3, and 6 after immunization, approximately 1% of CD8αα T cells were observed in the spleen (Fig. 6A). This value was not very different from that observed after usual immunization. Approximately 2%–3% of the cells were CD8αα+ within TCRαβ+CD8αβ− T cells (Figure 1A and data not shown). Significantly larger populations of CD8αα+ T cells were observed in PPs, IELs, and lamina propria lymphocytes on day 6 (Fig. 6B). In contrast, hardly any CD8αα+ T cells were observed in the gut on day 3 (Fig. 6B). Thus, these results suggested that differentiation of CD4 T cells into CD8αα T cells occurred at the site of immune reactions and that newly generated CD8αα T cells migrated into the gut through the expression of gut-homing receptors, as previously indicated[26,36]. In a previous study, we found that Runx3$^{-/-}$ mice showed defects in recovery from colitis and thereby increased the formation of inflammation-associated tumors[40]. We speculated that differentiated CD8αα T cells attenuate immune reactions and decrease the harmful effects of continuous inflammation, given that CD8αα Tregs are known to suppress activated T cells specifically. For a more detailed assessment of this hypothesis, we used mice with EAE as a model system. In this model, CD8 T cells play a key role in reducing the frequency of relapse after recovery from acute EAE[41,42]. This autoimmune disease is accordingly a suitable model for assessing the regulatory functions of CD8 Tregs. As expected, no differences in onset, clinical scores in the early phase, or EAE incidence were observed between genotypes. However, Runx3$^{-/-}$ mice exhibited defects in recovery from EAE, as indicated by the increased clinical scores after 6 weeks ($P = 0.0010$; Fig. 7A). To exclude effects derived from other lineage cells, we performed EAE-induction experiments using Rag2$^{-/-}$ mice, having transferred CD4 T cells from Runx3$^{-/-}$ and wild-type mice. Again, Rag2$^{-/-}$ mice having Runx3$^{-/-}$ CD4 T cells showed defects in recovery from EAE ($P = 0.0048$; Fig. 7B). These results indicate that Runx3 functions in CD4 T cells are important for recovery from EAE. Various CD4-derived cells are involved in EAE regulation: Th17, Th1, and CD4+Foxp3+ Treg cells. Th17 and Th1 are important for the induction of EAE disease. However, Runx family transcription factors are positive regulators for Th1 and Th17 differentiation, indicating that Runx3 deficiency will contribute to inhibiting EAE disease. Furthermore, Treg cell differentiation and function are not strongly affected by Runx3 deficiency, as shown previously[40,48]. In sum, the defect in recovery from EAE disease observed in Rag2$^{-/-}$ mice having Runx3-deficient CD4 T cells cannot be explained by previously known functions of Runx3 in CD4 T cells. For these reasons, we concluded that CD4-derived CD8αα T cells function as immune regulatory cells, at least in this EAE model. These observations support the hypothesis that differentiated CD8αα T cells play an important regulatory role in the establishment of a negative feedback loop that decreases adverse effects during immune reactions.

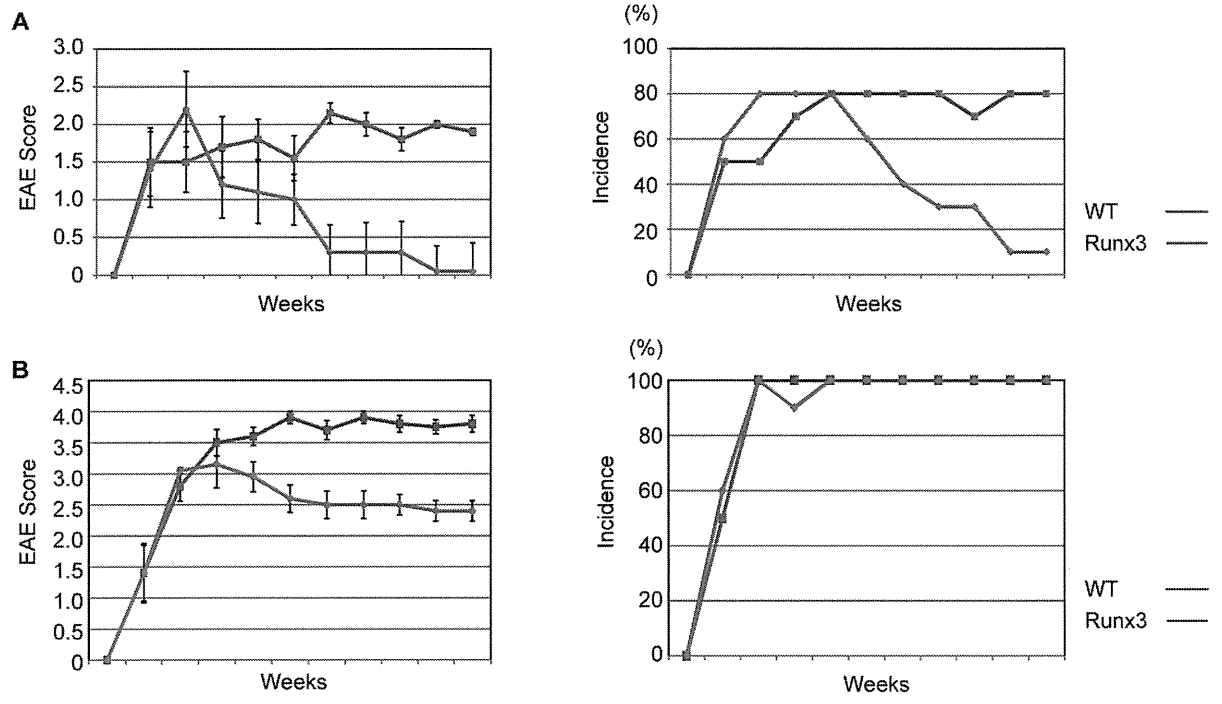

Figure 7 | Runx3$^{-/-}$ T cells cause defect in recovery from EAE. Rag2$^{-/-}$ mice with (A) WT or Runx3$^{-/-}$ lymphocytes and (B) WT or Runx3$^{-/-}$ CD4 T cells were followed up for signs of neurological disease after experimental autoimmune encephalomyelitis (EAE) induction. Data shown are mean ± standard error of mean of EAE clinical score of 10 mice pooled from two independent experiments and percentage disease incidence.

This study revealed previously unknown CD8 T-cell subsets that were derived from activated peripheral CD4 T cells (Fig. 8) and a unique mechanism for generating activated CD8 T cells without other CD4 T cells. Furthermore, these T-cell subsets can likely inhibit immune reactions. The elucidation of the precise mechanism of these processes and the exact functions of these cell subsets will provide fundamental insights for future autoimmune disease therapies.

Discussion

CD4 T cells are known to differentiate into various helper and regulatory T-cell lineages, namely Th1, Th2, Th9, Th17, T_{FH}, and CD4+Foxp3+ Tregs. In addition to these subsets, we identified a new cell population in this study called CD8αα Tregs, which have a regulatory function. Differentiation of CD8αα Tregs required the same signals as iTregs. However, the differentiation of CD8αα Tregs took more time than that of iTregs, and stronger TCR stimulation and higher atRA concentrations were required for their generation. These data indicate that the determination of the cell fate of one of the two inducible Treg cell subsets was differentially controlled during immune reactions. Therefore, iTregs and CD8αα Tregs may have distinct functions in immune regulation. According to this concept, some CD8αα T-cell subsets have been shown to have the property of inhibiting activated, but not naïve, T cells. Qa-1 in mouse (or HLA-E in human), which is a nonclassical MHC class I molecule, has been believed to play a key role in restricting the activity of CD8αα Tregs only toward activated T cells because these molecules are transiently upregulated on the surface of activated T cells and antigen-presenting cells. The correlation between CD8 Tregs and Qa-1 was suggested on the basis of previous studies as follows. Immunization with Qa-1-expressing activated CD4 T cells induces Qa-1-restricted CD8 Tregs[43,44]; CD8αα Treg clones recognize a TCR-derived peptide presented on Qa-1[11]; Qa-1$^{-/-}$ mice show augmentation of CD4 T-cell responses and increased susceptibility to EAE[45]; resistance to the re-induction of EAE was lost in CD8- and Qa-1-deficient mice[41,42,45,46]. In this study, we observed defects in recovery from EAE in Runx3-deficient mice in which CD4 T cells could not differentiate into CD8αα Tregs. These data indicate that CD8αα Tregs play a special role in the regulation of activated T cells. However, the molecular mechanisms underlying the suppressor functions of CD8αα Tregs are poorly understood. Furthermore, the exact target cells of CD8αα T cells and the relationship between CD8αα Tregs and Qa-1 in various immune reactions remain to be studied[47].

We found striking similarities between signals for CD4 and CD8αα Tregs. In addition, differentiation of these two regulatory T-cell subsets requires Runx protein activities; CD8αα Tregs require Runx3 and CD4 Tregs require Runx1[48]. Whether the different functions of Runx1 and Runx3 or the total amount of Runx proteins influence the determination of cell fate remains to be clarified.

CD8αα T cells are major populations of IELs, and most of them are selected in the thymus. The commitment of CD8ααTCRαβ T cells occurs from both MHC class I- and class II-restricted T cells[16,17]. Moreover, CD8αα IELs are absent in β2-microglobulin-deficient mice but not in MHC class Ia-deficient mice[49,50]. From these observations, MHC class Ib molecules, including Qa-1, are believed to be required for the selection of CD8αα T cells. In this study, we showed that CD4 T cells differentiated into CD8αα T cells in the periphery, indicating that MHC class II-dependent primary selection of these cells occurred in the thymus. It is of interest to determine the exact roles of the Qa-1 molecule in the differentiation, maintenance, or expansion of CD4-derived CD8αα T cells.

In summary, we have identified a new subset of inducible Treg cells (Fig. 8). This differentiation system can be affected by various immune dysfunctions, including autoimmune diseases and tumor immunity. We anticipate that further elucidation of the precise mechanisms of this process will provide a basis for development of anti-cancer drugs and a rationale for treatment of autoimmunity.

Methods

Mice. This study received appropriate ethics approval from the Institutional Review Board of Kyoto University (Reference Number: Med Kyo12076). Wild-type (WT), Runx3$^{+/-}$, Runx2$^{+/-}$ and Rag2$^{-/-}$ mice (CD45.1) in a C57BL/6 genetic background were maintained in a specific pathogen-free mouse facility. Procedures involving

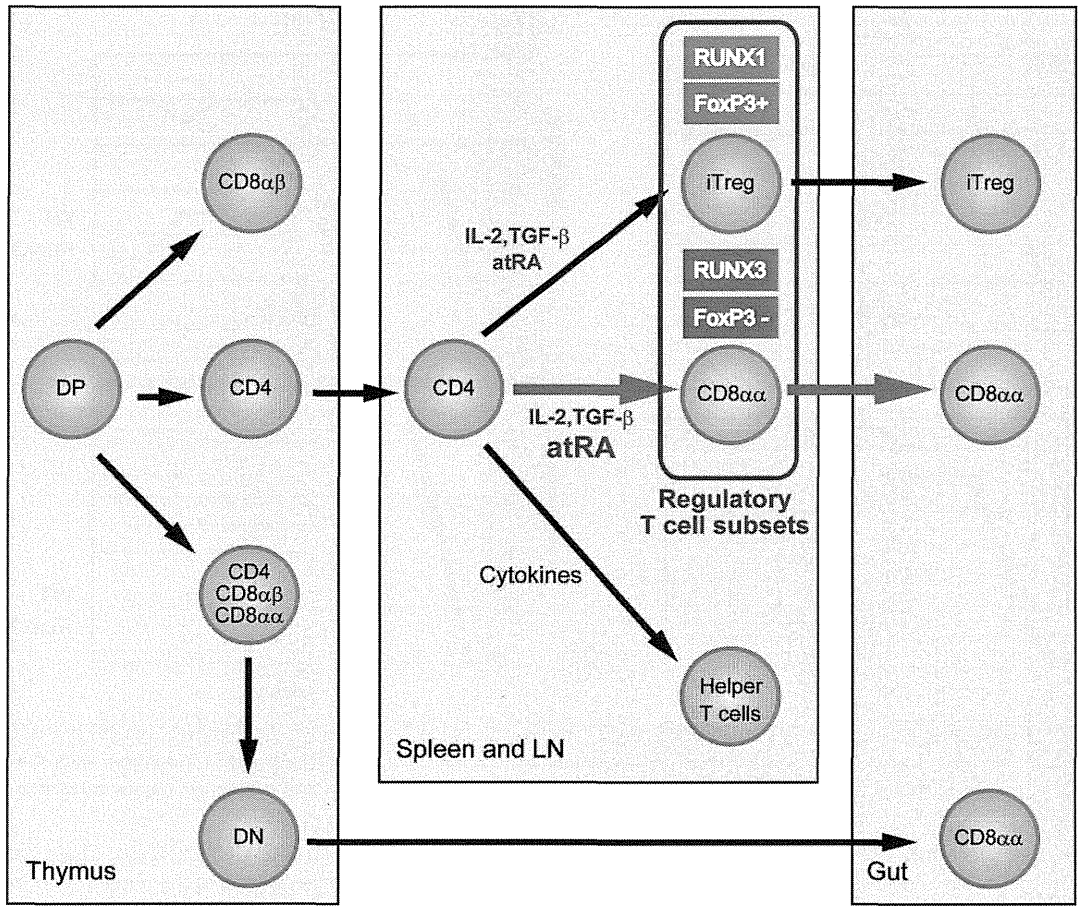

Figure 8 | Schematic of CD8αα T cell differentiation pathways.

animals and their care were in accordance with the guidelines for animal treatment of the Institute of Laboratory Animals, Kyoto University.

Fetal liver transfer. Single-cell suspensions of 2–4 × 10^6 whole fetal liver mononuclear cells harvested from Runx3$^{-/-}$, Runx2$^{-/-}$Runx3$^{-/-}$and WT (CD45.2) embryos at E14.5 were injected intravenously into sublethally irradiated (4 Gy) Rag2$^{-/-}$ recipient mice (CD45.1). At least 10 weeks after transplantation, mice were used for EAE induction or were killed, and their naïve CD4 T cells were analyzed by flow cytometry or used for *in vitro* culture. In some experiments, naïve CD4 T cells were purified and adoptively transferred into other Rag2$^{-/-}$ recipient mice (CD45.1). All Rag2$^{-/-}$ (CD45.1) mice used for each experimental set were age- and sex-matched.

Cell preparation. Naïve CD4+TCRαβ+ was prepared by magnetic cell sorting (MACS) using a CD4+ CD62L+ T cell isolation kit (Miltenyi Biotec, Bergisch, Germany) in combination with various antibodies (BD Pharmingen). Briefly, lineage positive cells, but not CD4, were removed according to the manufacturer's instruction using biotin-conjugated lineage-specific antibodies and α-Biotin microbeads. After negative selection, we made a rough prediction of the final concentration of contaminated CD4-CD62L+ cells after purification of CD4+CD62L+ cells. For this purpose, a part of the negatively selected cells were stained with α-CD4 and α-CD62L antibodies. If more than 5% cells were CD4-CD62L+ among CD62L+ cells, we performed negative selection again using the following antibodies and magnetic beads; α-CD8α-FITC, α-CD25-Biotin, α-CD19-Biotin, α-B220-Biotin, α-CD11b-Biotin, α-CD11c-Biotin, α-Ia-Biotin, α-Dx5-Biotin, α-TCRγδ-Biotin, Ter119-Biotin (*Becton Dickinson, Mountain View, CA*), α-FITC microbeads and streptavidin microbeads (Myltenyi Biotech Bergisch, Germany). After the second round of negative selection was finished, CD62L+ cells were further selected according to the manufacturer's instructions. Purities were determined by staining with antibodies for CD8α, CD8β, CD4 and CD62L. Usually, more than 97% cells were CD4+CD62L+ and fewer than 0. 02% cells were CD8α+. CD8αα cells cannot be detected. If more than 0.02% cells were positive for CD8α expression or fewer than 97% cells were CD4+CD62L+, we further purified CD4+CD62L+ cells by flow cytometry using FACSAria. All the purified cells using the above method were used for both *in vitro* culture and cell transfer experiments into RAG2$^{-/-}$ mice. Essentially all the methods of purification were used for *in vitro* culture and cell-transfer experiments.

For purification of CD4 T cells, spleen cells were subjected to negative selection using the following antibodies and magnetic beads; α-CD8α-FITC, α-CD19-Biotin, α-B220-Biotin, α-CD11b-Biotin, α-CD11c-Biotin, α-Ia-Biotin, α-Dx5-Biotin, α-TCRγδ-Biotin, Ter119-Biotin (Becton Dickinson), α-FITC microbeads and streptavidin microbeads.

CD8αβ+TCRαβ+ T cells was prepared by MACS using a CD8α+ T cell isolation kit (Miltenyi Biotec) in combination with various antibodies (BD Pharmingen). Briefly, lineage positive cells, but not CD8, were removed according to the manufacturer's instruction using biotin-conjugated lineage-specific antibodies and α-Biotin microbeads. Then we further performed second round of negative selection using the following antibodies and magnetic beads; α-CD4-Biotin, α-CD19-Biotin, α-B220-Biotin, α-CD11b-Biotin, α-CD11c-Biotin, α-Ia-Biotin, α-Dx5-Biotin, α-TCRγδ-Biotin, Ter119-Biotin (Becton Dickinson), and Streptavidin microbeads. After a second round of negative selection, CD8β cells were positively selected by MACS. Usually more than 98% of populations were CD8αβ+ cells and fewer than 0.5 % were CD8αα+ cells. If fewer than 96% cells were CD8αβ+, or more than 1.0% were CD8 αα+ cells, we further purified CD8αβ+ cells by flow cytometry using FACSAria. All the purified cells using above method were used for both *in vitro* culture or cell transfer experiments into RAG2$^{-/-}$ mice.

Transfer of CD4 T cells or naïve CD4 T cells. Naïve CD4 T cells were prepared as described above; 0.5–2.5 × 10^6 naïve CD4 T cells (CD45.2) were transferred into Rag2$^{-/-}$ (CD45.1) mice. At least 4 weeks after transplantation, the mice were used for various experiments. For EAE experiments, 2.5 x10^6 CD4 T cells (CD45.2) were transferred into Rag2$^{-/-}$ (CD45.1) mice. Several days after transplantation, mice were used for EAE induction.

Cell cultures. In all experiments, the percentages of CD62L+CD4+ T cells or CD8αβ+ T cells were above 97. Naïve CD4+ T cells (1 × 10^5 cells /mL) were activated with plate-bound anti-CD3 (5 μg/mL; BD Pharmingen), soluble anti-CD28 (1 μg/mL; BD Pharmingen), anti-interferon-γ (3 μg/mL; BD Pharmingen), anti-IL-4 (3 μg/mL; BD Pharmingen), TGF-β1 (3 ng/mL; R&D) and IL-2 (20 ng/mL; R&D) in the presence or absence of atRA (10 nM–10 μM; Sigma-Aldrich) for 7–9 days.

RA injection. atRA (Sigma-Aldrich, Saint Louis, MO) was dissolved in soybean oil at 6 mg/mL and aliquots were stored at −80°C. A working solution was prepared by diluting the atRA aliquots in soybean oil and an equal volume of dimethyl sulfoxide

(DMSO). atRA of 200 μg/100 μL, 600 μg/300 μL or vehicle was delivered by intraperitoneal injection 1, 3, and 6 d before analysis.

Flow cytometric analysis. The following antibodies were used for staining: PECy7 anti-CD45.2 (BD Pharmingen); allophycocyanin (APC) anti-mouse TCRαβ (BD Pharmingen); APCCy7 anti-mouse CD8α (BD Pharmingen); fluorescein isothiocyanate (FITC) anti-mouse CD8β (BD Pharmingen); phycoerythrin (PE) anti-mouse CD4 (BD Pharmingen); PE anti-mouse CD8α (BD Pharmingen); APC anti-mouse CD4 (BD Pharmingen); FITC anti-mouse CD8α (BD Pharmingen); FITC anti-mouse CD4 (BD Pharmingen); PE anti-mouse CD44 (BD Pharmingen); Biotin anti-mouse CD122 (BD Pharmingen); APC anti-mouse CXCR5 (BD Pharmingen); Biotin anti-mouse α4β7 (BD Pharmingen); FITC anti-mouse CCR9 (R&D); PE anti-mouse Granzyme A (Santa Cruz CA); Alexa 647 anti-Foxp3 (BD Pharmingen); Alexa 647 anti-Granzyme B (BD Pharmingen); PerCP–streptavidin (Molecular Probes, Eugene, OR); APC–streptavidin (Molecular Probes, Eugene, OR); APCCy7–streptavidin (BD Pharmingen); PECy7–streptavidin (BD Pharmingen); PE–streptavidin (Molecular Probes); FITC–streptavidin (Molecular Probes).

For cytoplasmic staining, BD Cytofix/Cytoperm solution and BD Perm/Wash solutions (BD Pharmingen) were used.

All analyses were performed with FACSCalibur or FACSAria (Becton Dickinson).

IELs and LPLs preparation. To prepare IELs and LPLs, the intestines were removed, and PPs and mononuclear lymphocytes were isolated. The intestines were opened longitudinally, washed with phosphate-buffered saline, and cut into 5-mm pieces. To obtain IELs, pieces were shaken in Hank's balanced salt solution (+) containing 5 mM ethylenediaminetetraacetic acid for 20 min at 37°C and passed through a cell strainer twice. The remaining tissue was cut into smaller pieces and digested with RPMI 1640 medium containing 4% fetal calf serum, 1 mg/mL collagenase II, 1 mg/mL Dispase (Gibco), and 40 μg/ml DNase I (TaKaRa) at 37°C and stirred for 20 min. Whole intestinal cell suspensions, which included LPL or IEL, were passed through a cell strainer and loaded on a 40/80% discontinuous Percoll gradient (GE Healthcare), and cells at the interface between 40% and 80% were collected and used as IELs or LPLs.

Immunization. 100 μg NP- chicken gamma globulin (CGG) in alum or complete Freund's adjuvant (incomplete Freund's adjuvant with *Mycobacterium tuberculosis* H37RA; Difco) was intraperitoneally injected.

EAE induction and clinical scoring. Age- and sex-matched mice were immunized subcutaneously with 100 μg MOG35-55 (Genway, China) in IFA (incomplete Freund's adjuvant) supplemented with 500 μg *Mycobacterium tuberculosis* on day 1. On days 1 and 3, mice were injected with 300 ng pertussis toxin (List Biological Laboratories). Clinical signs were scored on a scale of 1–5: 0, no clinical sign; 0.5, partially limp tail; 1, paralyzed tail; 2, loss of coordinated movement, hind limb paresis; 2.5, one hind limb paralyzed; 3, both hind limbs paralyzed, 3.5, hind limbs paralyzed, weakness in fore limbs; 4, fore limbs paralyzed; and 5, moribund.

Reverse transcription polymerase chain reaction (RT-PCR). Total RNAs were extracted from sorted T cells using TRIzol reagent (Gibco-BRL, Gaithersburg, MD). Oligo (dT)-primed cDNAs were prepared by reverse transcription. For semiquantitation, 50 ng cDNA was serially diluted and subjected to PCR. All PCR products were resolved electrophoretically on 2% agarose gels and visualized by ethidium bromide staining.

Statistical analysis. Proportions of cells were compared with Student's *t* test (Fig. 1A). To see the effect of Runx3 in EAE recovery, the mean clinical scores were compared between genotypes using repeated measures analysis of variance (ANOVA). Greenhouse–Geiser correction was applied to adjust the degree of freedom for deviation from the sphericity assumption (Fig. 7A). All reported *P* values were two-tailed and *P* values lower than 0.05 were considered to indicate statistical significance.

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Author contributions

Y.N. and M.S. performed the experiments; T.H., K.-J.J., K.N., H.M., K.A. and K.T. assisted with experiments; M.S. designed and conceptualized the study; Y.I. and T.K. provided critical materials; S.T. performed statistical analysis; Y.N., K.A., M.O., K.I. and J.F. made comments; A.S. , Y.N. and M.S. interpret the data; and M.S. wrote the manuscript.

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An exploratory clinical study on the safety and efficacy of an autologous fibroblast-seeded artificial skin cultured with animal product-free medium in patients with diabetic foot ulcers

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ABSTRACT

Cultured dermal substitutes have been used for the treatment of chronic skin ulcers; however, the biological risks of animal-derived materials in the culture process such as foetal bovine serum (FBS) have been reported. In this study, we prepared an autologous fibroblast-seeded artificial dermis (AFD) using animal product-free medium supplemented with 2% patient autologous serum and without any animal-derived materials such as trypsin in the culturing process. We applied AFD in five patients with diabetic ulcers and investigated its safety and efficacy. As the primary endpoint, we defined, 'wound bed improvement' according to the percentage of granulation area to the whole wound area on day 21, and 60% or higher was regarded as improved. The mean age of the patients was 60·6 years and the mean duration of the ulcer was 22·6 months. In the evaluation of the primary endpoint, the 'wound bed' was improved in all patients [proportion of improvement: 100%, 95% confidence interval (CI): 48% to 100%]. Three patients had complete wound healing within 12 weeks after application and two patients had >80% wound healing at 12 weeks. Side effects were not serious. Our AFD may be a safe and effective treatment of diabetic ulcers.

Key words: Animal product-free medium • Autologous fibroblast-seeded cultured dermal substitute • Autologous serum • Diabetic foot ulcer

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Key Points

- we prepared an autologous fibroblast-seeded artificial dermis (AFD) using animal product-free medium supplemented with 2% patient autologous serum and without any animal-derived materials such as trypsin in the culturing process
- we applied AFD in five patients with diabetic ulcers and explored the safety and efficacy of AFD

INTRODUCTION

Diabetic foot ulcer, mainly because of neuropathy and arterial occlusive disease, is one of the most common complications of diabetic patients and is a leading cause of major amputations of the lower limbs. Throughout the world, over 250 million people are reported to be suffering from diabetes mellitus and the number still increasing (1). The prevalence of foot ulcers ranges from 4% to 10% among patients with diabetes mellitus (2,3) with an annual population-based incidence of 1% to 5% (1–3), and the lifetime incidence may be as high as 25% (2,3). More than 15% of all ulcers result in some form of amputation (2,3). In the treatment of diabetic foot ulcers, it is necessary to evaluate and improve the blood supply if needed to reduce the ulcer and to perform appropriate local ulcer treatment (1–3). Recently, several new wound therapies have been developed based on the understanding of the wound-healing process and cell technology (4,5). Among these new therapies, tissue-engineered skin substitutes have made marked advances in the field of wound healing and the treatment of chronic wounds such as diabetic ulcers (6–9). Generally, most of the cells used for these skin substitutes are derived from allogeneic neonatal foreskin, and a cocktail of cytokines and growth factors secreted from living skin substitutes is considered to promote wound healing (8–10).

We developed bilayered acellular artificial skin composed of an upper silicone sheet and a lower collagen sponge (11) by modifying the material described by Yannas and Burke (12). In our previous study, we reported that an autologous fibroblast-seeded artificial dermis (AFD) promoted the epithelisation of wounds and prevented wound contraction compared with an allogeneic fibroblast-seeded collagen sponge (13). In the conventional culturing process of cultured skin substitutes, foetal bovine serum (FBS) has been used as a nutrient source (8,9); however, the usage of FBS raises safety concerns because it might transmit prion or viral diseases (14,15) and cause unfavourable immune and local inflammatory reactions to bovine proteins (16). In our previous study, we reported that animal product-free medium (HFDM-1; Cell Science & Technology Institute, Inc., Miyagi, Japan), which is animal component-free medium for human fibroblasts, supplemented with 2% autologous serum could expand autologous primary fibroblasts and those after seeding to collagen sponges as well as Dulbecco's modified eagle medium (DMEM) with 10% FBS (17).

In this study, we prepared AFD cultured using HFDM-1 supplemented with 2% patient autologous serum and without using animal-derived materials such as trypsin in the culturing process. We then investigated the safety and efficacy of our AFD in the treatment of diabetic ulcers.

MATERIALS AND METHODS

Study design

This study was a prospective, open-labelled, proof-of-concept clinical trial conducted in Kyoto University Hospital. Patient registration and data management were performed in an independent data center at the Translational Research Center, Kyoto University Hospital. The study protocol was approved

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by the ethics committee of Kyoto University Graduate School and Faculty of Medicine and was conducted according to the Declaration of Helsinki.

The eligibility criteria were chronic (at least 1-month duration), non healing (without granulation tissue and skin graft not expected to take), full-thickness, lower extremity ulcers resulting from diabetes mellitus, at least 20 years of age, adequate arterial blood supply assessed by measuring skin perfusion pressure (SPP) of 25 mmHg or greater at a site of 1 cm proximal or distal to those ulcers. Written informed consent was obtained.

The exclusion criteria were systemic infection including hepatitis B, hepatitis C, human immunodeficiency virus and syphilis; ulcers with local infection based on clinical examination, ulcer larger than 70 × 50 mm^2; ulcers with large tendon or bone exposure; osteomyelitis and cellulitis; malignant disease; uncontrolled diabetes mellitus (defined by HbA1c ≥12%) or requiring continued use of oral corticosteroid therapy (> 5 mg/day prednisolone equivalent); patients receiving immunosuppressive agents; pregnant women; nursing mothers and patients with hypersensitivity to any component of AFD or local anaesthetics and antiseptics.

Preparation of autologous fibroblast-seeded AFD

After enrollment, a skin biopsy (1 cm × 5 mm) and 20 ml blood were taken from each patient. All cell processing procedures for autologous fibroblasts and fibroblast-seeded AFD were performed in the Center for Cell and Molecular Cell Therapy, which is a cell processing laboratory following current good manufacturing practices in Kyoto University Hospital. Patient autologous serum was also prepared, and blood was taken again if needed. In the culturing process, no animal-derived material was used. The skin specimens were cut into small pieces with surgical scissors, placed in a 10-cm tissue culture dish and cultured using HFDM-1 supplemented with 2% autologous serum. Outgrowing fibroblasts were dissociated using TripLE Select (Life Technologies Co., Carlsbad, CA) and passaged. The fibroblasts were seeded onto AFD (Pelnac S size: 82 × 60 mm^2; Gunze Ltd., Kyoto, Japan) at a density of $1{\cdot}0 \times 10^5$ cells/cm^2 and cultured for 10 days before grafting.

Autologous graft treatment and subsequent therapy

The ulcer was surgically debrided to remove necrotic tissue and chronic granulation tissue. AFD was then cut according to the shape of the wound, grafted onto the debrided wound and sutured to the marginal skin. AFD was covered with non adherent paraffin and framycetin gauze (Sofratulle; Sanofi-Aventis K.K., Tokyo, Japan) and sterile cotton pads and gauze. This study used a single application of AFD. After the application of AFD, dressings were changed as necessary. No topical agent accelerating wound healing, such as basic fibroblast growth factor (bFGF) spray (18) (bFGF spray and Fibrast spray; Kaken Pharmaceutical, Tokyo, Japan) was applied until day 21 after AFD application. The use of antibiotic ointment and dressing was allowed. Patients were hospitalised until day 21 to ensure stabilisation of the applied AFD. On day 21 after application, the sutures and silicone sheet of AFD were removed, and subsequent therapy including skin graft and any topical agent could be started. Patients were followed up until 12 weeks after application to observe the adverse events related to the application of AFD.

Evaluation of treatment

Using a digital camera, digital images of the wounds were taken with a calibrator (CASMATCH; BEAR Medic Corp., Tokyo, Japan) placed on the skin adjacent to the wound at enrollment, before grafting, just after grafting and 3, 5, 7, 9 and 12 weeks after grafting. The colour and size of images were adjusted using CASMATCH and image editing software (Adobe Photoshop CS4; Adobe Systems Inc., CA) to assess the wound and granulation areas. As with the primary endpoint, the granulation area on day 21 was independently measured under blinding by a central review.

The primary endpoint in this study was, 'wound bed improvement on day 21'. Granulation tissue is wound connective tissue, which forms at the beginning of wound healing (19). This highly fibrous tissue is usually pink because numerous small capillaries invade granulation tissue to supply oxygen and nutrients. The appearance of granulation tissue is a good sign of healing because it means that

Table 1 Baseline data of patients and outcome

Case	Age	Sex	Complications	Lesions	Before application			21 days after application			12 weeks after application
					Duration of ulcer (months)	Area of ulcer (cm^2)	SPP (mmHg)	Area of ulcer (cm^2)	Granulated area (cm^2)	Wound improvement (%)	Healing
1	51	M	CRF on HD, DR	Right foot	75	7·46	104·5	53·88	2·7	70	Improved	90% Reduction of wound size with skin graft.
2	65	M	CRF on HD, PMR, DR	Left foot	16	0·7	62	0·3	0·3	100	Improved	Complete wound closure without skin graft.
3	47	M	CRF on HD, DR	Left foot	5	9·2	59·5	5·6	4·6	82	Improved	80% Reduction of wound size with skin graft.
4	67	M	CRF, DR	Right foot	13	4·3	75	1·1	0·9	82	Improved	Complete wound closure without skin graft.
5	73	M	CRF, DR, OMI	Left foot	4	13·7	74·5	6·2	4·8	77	Improved	Complete wound closure without skin graft.
Mean	60·6				22·6	7·07	7·51	3·42	2·66	82·2		

SPP, skin perfusion pressure; DM, diabetes mellitus; CRF, chronic renal failure; HD, haemodialysis; PMR, polymyalgia rheumatica; DR, diabetic retinopathy; OMI, old myocardial infarction.

the healing process of the wound is starting (19–22). The area of granulation tissue was measured in this study, and the percentage of wound bed improvement was defined as the value (%) calculated from the granulation areas on day 21 divided by the wound area on day 21 multiplied by 100, and the wound bed was regarded as improved when the value was 60% or higher. Although a value of 50% or more as the cutoff for wound bed improvement was proposed by the Japanese Society of Pressure Ulcers (21–23), we used 60% as a stricter cutoff value because it is sufficient to show that the wound has begun to heal.

The secondary endpoints were the area of target ulcers on day 21, time to complete wound closure, time to skin grafting, area of the skin graft after 2 and 4 weeks and adverse events.

Statistical analysis

This study was designed to test the hypothesis that the wound bed improvement rate on day 21 is >5%. We calculated that 11 patients were required for a power of 90% with a one-sided alpha of 5% based on the one-sided binomial test with a null hypothesis of 5% and alternative hypothesis of 35%. Two interim analyses were planned using five and eight patients for safety and efficacy. Changes in the mean wound area from just after application of AFD to day 21 were examined by the paired *t*-test. All patients were included in the efficacy and safety analyses. All data management and statistical analysis were conducted in the Department of Clinical Trial Design and Management, Translational Research Center with the use of SAS version 9.2 (SAS Institute, Cary, NC).

RESULTS

Five patients with diabetic ulcers were enrolled in the study between September 2008 and December 2009 and included in the first interim analysis. The baseline data of patients, outcome of the primary endpoint and wound healing at 12 weeks are shown in Table 1. All patients were male and the mean (±SD, standard deviation) age was 60·6 (±11·1) years old. The mean duration of ulcer was 22·6 (±26·6) months. In all patients, AFD could be produced using HFDM-1 supplemented with 2% autologous serum. All patients received a single application of AFD and

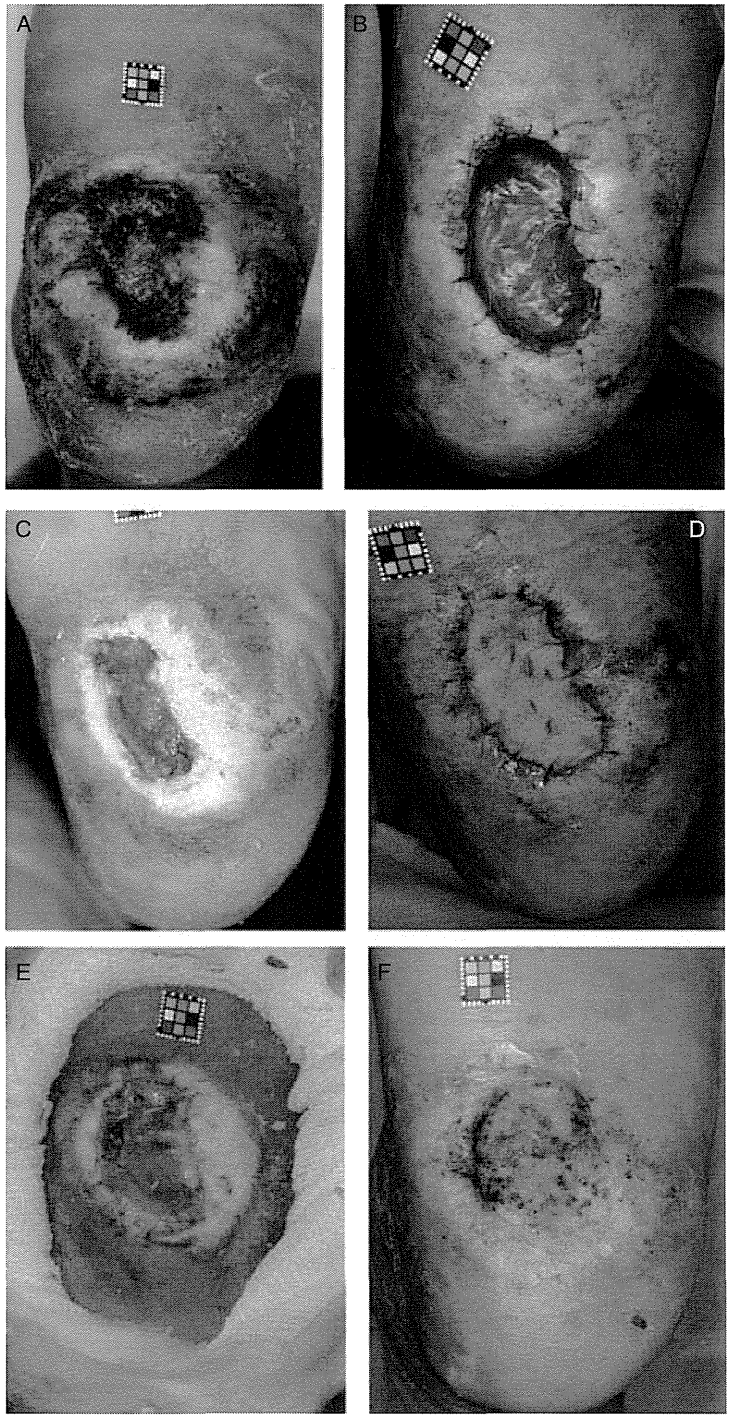

Figure 1. A case of diabetic ulcer of the right heel (Case 1). (A) The ulcer area was 7·46 cm^2. (B) AFC was applied and sutured to the marginal skin after the third surgical debridement. (C) By 21 days after application, granulated tissue had formed in 70% of the wound area. (D) 28 days after application, a full-thickness skin graft was applied. (E) By 14 days after skin grafting, the central part of the graft had taken on the wound bed. (F) 12 weeks after application, the ulcer was nearly healed.

were followed up until 12 weeks after application. In the evaluation of the primary endpoint, granulation tissue had formed over >70% of the wound area on day 21 and the 'wound bed' was improved in all five patients [proportion of improvement: 100%, 95% confidence interval (CI): 48% to 100%]. This number of patients with wound bed

improvement exceeded the number required for significance in the planned final analysis of 11 patients. Regarding a secondary endpoint, the mean wound area ($3{\cdot}42 \pm 2{\cdot}36$ cm^2) on day 21 was significantly reduced compared with the area ($7{\cdot}07 \pm 4{\cdot}92$ cm^2) just after application of AFD ($P = 0{\cdot}03$). The wounds of three patients healed completely during the trial (proportion of complete wound closure: 60%, 95% CI: 15% to 95%). Only one patient underwent skin grafting on day 29 and the area of the skin graft was 46% at 2 weeks and 17% at 4 weeks.

There were 30 adverse events in the five patients. One serious adverse event (reflux esophagitis) was regarded as not being related to the AFD. The most frequent adverse events included hypoglycemic attack, nausea and vomiting. Two adverse events (one wound infection and one wound pain) may have been related to the products used in the study, but they recovered quickly.

Interim analyses of the five patients showed that the AFD was safe, with promising efficacy, and we therefore stopped this study.

Case presentations

A 51-year-old man (case 1) with a diabetic ulcer on his right heel was enrolled in this trial (Figure 1A). The duration of his ulcer was 75 months and SPP at a site 1-cm proximal to the ulcer was 105 mmHg. Skin ($10\times$ 5 mm^2) from the groin region and 20 ml of blood were taken, and autologous fibroblasts and AFD were prepared. In the culturing process, 11 ml of autologous serum was used and the culture period was 46 days. The necrotic tissue was debrided three times under local anaesthesia and after the third debridement, AFD was applied to the wound (Figure 1B). Twenty-one days after application, the granulation area was evaluated as 70% of the wound area and the 'wound bed' was regarded as improved (Figure 1C). Four weeks after application, full-thickness skin was grafted (Figure 1D) and the central part of the graft took (Figure 1E). This ulcer had healed nearly 12 weeks after application (Figure 1F).

A 67-year-old man (case 4) had a stump ulcer for 13 months after right transmetatarsal amputation because of gangrene (Figure 2A). SPP at a site 1 cm proximal to the ulcer was 75 mmHg. In the culturing process in this case, the amount of autologous serum was 11 ml and culture period was 46 days. After debridement, AFD was applied (Figure 2B). Twenty-one days after application, the granulated area was evaluated as 82% of the wound area and the 'wound bed' was regarded as improved

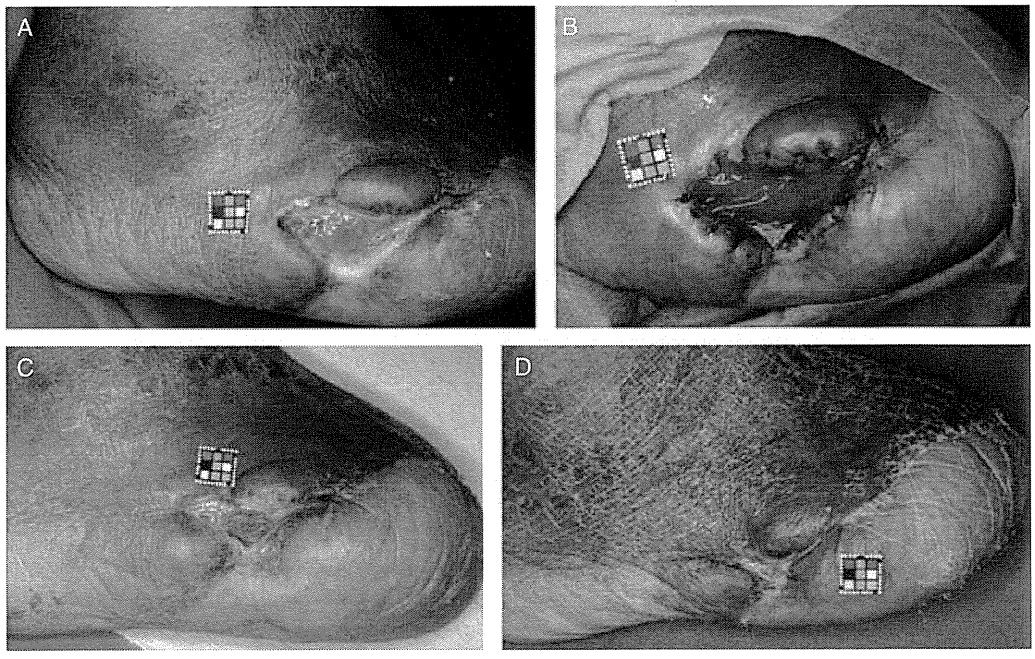

Figure 2. A case of stump ulcer after transmetatarsal amputation (Case 4). (A) The ulcer area was 4·3 cm^2. (B) Artificial dermis was applied and sutured to the marginal skin after debridement. (C) By 21 days after application, the ulcer was filled with granulation tissue. (D) By 9 weeks after application, the ulcer had healed completely.

(Figure 2C). This ulcer healed completely 77 days after application (Figure 2D).

DISCUSSION

A bilayered living human skin equivalent (Apligraf; Organogenesis Inc., Canton, MA) and a human fibroblast-derived dermal substitute (Dermagraft; Advanced BioHealing Inc., La Jolla, CA) are representative bioengineered products and have been reported to be effective for the treatment of diabetic foot ulcers (8,9,24). Autologous dermal and epidermal tissue-engineered grafts (Hyalograft 3D autograft and Laserskin autograft, respectively; Anika Therapeutics srl, Abano Terme, Italy) have been used to treat chronic ulcers and it was reported that a 50% reduction in the diabetic ulcer area was achieved significantly faster in the treatment group (6). The complete ulcer healing rate at 12 weeks was reported to be 24% to 56% (24–29). In this study, three of the five patients showed complete healing at 12 weeks, indicating that the effectiveness of our AFD was comparable with that of skin equivalent products.

As the primary endpoint in this study, we defined, 'wound bed improvement on day 21' according to the granulation area. Skin equivalent products are usually applied once or twice a week until complete wound healing. In this study, AFD was applied only once in consideration of safety because this was the first clinical trial of our AFD; therefore, we needed to assess whether hard to heal ulcers would heal as do acute wounds. We assessed all cases as 'wound bed' improved and 60% healed within 12 weeks, so this index may be used to predict the probability of wound healing.

FBS has been used in the culture process of conventional products of dermal substitutes. Allogeneic human serum can be used for the culture of fibroblasts as an alternative to FBS, but the possibility of disease transmission and immune reactions remains; therefore, the use of patient autologous serum is desirable to avoid the risk of problems associated with FBS. Diabetic patients usually have systemic complications such as a poor nutritional condition or chronic renal failure, and it is frequently difficult to take a large amount of autologous serum. In this study, the average amount of autologous serum needed for the preparation of AFD was 11·6 ml and this amount would not be an issue in diabetic patients, even those on haemodialysis.

Considerable efforts have been made to eliminate animal-derived materials from the culturing process, and several animal product-free media for mesenchymal stem cells have been reported (30). An animal product-free medium for fibroblast cultivation may be available in the near future.

In conclusion, we prepared autologous fibroblast-seeded AFD using HFDM-1 supplemented with 2% autologous serum without using animal-derived materials and applied it in the treatment of five diabetic ulcers. In this study, side effects were not serious and three patients were completely healed within 12 weeks after application. Although this study was a preliminary clinical trial with a small sample size, our dermal substitutes may provide a safe and effective treatment of diabetic ulcers.

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An eClinical trial system for cancer that integrates with clinical pathways and electronic medical records

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What is This?

CLINICAL TRIALS DATA MANAGEMENT AND STUDY CONDUCT Clinical Trials 2012; 9: 408–417

An eClinical trial system for cancer that integrates with clinical pathways and electronic medical records

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Background Various information technologies currently are used to improve the efficiency of clinical trials. However, electronic medical records (EMRs) are not yet linked to the electronic data capture (EDC) system. Therefore, the data must be extracted from medical records and transcribed to the EDC system. Clinical pathways are planned process patterns that are used in routine clinical practice and are easily applicable to the medical care and evaluation defined in a trial protocol. However, few clinical pathways are intended to increase the efficiency of clinical trials.

Purpose Our purpose is to describe the design and development of a new clinical trial process model that enables the primary use of EMRs in clinical trials by integrating clinical pathways and EMRs.

Methods We designed a new clinical trial model that uses EMR data directly in clinical trials and developed a system to follow this model. We applied the system to an investigator-initiated clinical trial and examined whether all data were extracted correctly. At the protocol development stage, our model measures endpoints based on clinical pathways with the same diagnosis. Next, medical record descriptions and the format of the statistical data are defined. According to these observations, screens for entry of data, which are used both in clinical practice and for study, are prepared into EMRs with an EMR template, and screens are prepared for data checks on our EMR retrieval system (ERS). In an actual trial, patients are registered and randomly assigned to a protocol treatment. The protocol treatment is executed according to clinical pathways, and the data are recorded to EMRs using EMR templates. The data are checked by a local data manager using reports created by the ERS. After edit checks and corrections, the data are extracted by the ERS, archived in portable document format (PDF) with an electronic signature, and transferred in comma-separated values (CSV) format to a coordinating centre. At the coordinating centre, the data are checked, integrated, and made available for a statistical analysis.

Results We verified that the data could be extracted correctly and found no unexpected problems.

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Limitation To execute clinical trials in our system, the EMR template and efficient ERSs are required. Additionally, to execute multi-institutional clinical trials, it is necessary to create templates appropriate for EMRs at all participating sites and for the coordinating centre to validate local templates and procedures.

Conclusion We proposed and pilot tested a new eClinical trial model. Because our model is integrated with routine documentation of clinical practice and clinical trials, redundant data entries were avoided and the burden on the investigator was minimised. The reengineering of the clinical trial process would facilitate the establishment of evidence in the future. *Clinical Trials* 2012; **9**: 408–417. http://ctj.sagepub.com

Background

To improve the efficiency of clinical trials, evaluating and restructuring the information-capture process and the flow of data are critical [1–3]. In many clinical trials, paper-based case report forms (CRFs) are developed for each clinical trial protocol and information is transcribed from source documents, including medical records, to CRFs and from CRFs to the clinical data management system (CDMS). Thus, mistranscriptions and inconsistencies are inevitable in this dual transcription process [2,3]. Various information technologies currently are used to improve the efficiency of clinical trials, including computerised registration systems, CDMS and electronic data capture (EDC) systems, among others [2,3,4]. Elsewhere, medical information technology recently has been promoted. The electronic medical record (EMR) system has been launched in approximately 20% of all institutions in Japan. However, EMRs are not yet linked to the EDC system for clinical research; therefore, the clinical trial staff must manually select the necessary information from EMRs and transcribe it into the EDC system [2]. Thus, the workload at each participating site has not changed with the transition from paper-based CRFs to EDC systems, and there has been no breakthrough in efficiency [2].

The president of the Clinical Data Interchange Standards Consortium (CDISC), Rebecca Daniels Kush, named the next generation of clinical trials 'eClinical trials' [3]. She emphasised the importance of optimising clinical trials by redesigning the trial process to utilise primarily electronic processes and to coordinate hospital information systems with the various clinical trial systems [3]. In addition, the CDISC's Electronic Source Data Interchange (eSDI) initiative, encouraged by the US Food and Drug Administration, was started in 2004 [5]. The eSDI detailed the regulatory requirements and outlined promising scenarios concerning clinical trials using electronic source data (eSource), such as electronic subject diaries, electronic laboratory results, and EMRs. To enhance clinical trials utilising eSource, the eSDI analysed the present regulatory requirements, compiled 12 indispensable requirements, outlined five scenarios that would fit the regulatory requirements, and formed a proposal.

Currently, most clinical trials including standard-based approaches that use data from EMRs are planned according to the concept that the primary use of EMRs is in clinical practice and a secondary use is in clinical research [6–11]. To use EMR data secondarily in a clinical trial, it is necessary to obtain various types of information that are stored in EMRs by category, such as the diagnosis and medications taken. However, extraction of this information is not facilitated by the current EMR system [11–13]. Moreover, not all information that is necessary to execute a clinical trial typically is stored in the EMR [6,10–12]. If EMRs are used only for the purpose of collecting certain receipts of claims and the treatments for each patient, enough information may be stored in the current EMRs. However, if there is a secondary intention to use the accumulated information from EMRs in clinical research, then the data that are not gathered specifically for research purposes may be incomplete and unreliable [6,11,12]. To improve the efficiency of clinical trials, it is necessary to establish a method that utilises EMRs directly in clinical trials and enables higher levels of information sharing and exchange between clinical practice and clinical trials [1–3].

Clinical pathways have been introduced in clinical practice [14,15]. Clinical pathways are planned process patterns aimed at improving both process quality and resource usage [14,15]. Clinical pathways include components such as a timeline, the categories of care or activities and the interventions for specified groups of patients with a particular diagnosis. They are widely used as a treatment plan or as a substitute for physicians' orders [14,15]. Clinical pathways appear to be easily applicable to the protocol treatment of a clinical trial because they deliver a standard of care to patients with a specific disease [14,15]. However, there are few clinical pathways that purport to improve the efficiency of clinical trials.

Purpose

Our purpose is to develop a new clinical trial process model that enables the primary use of EMRs in a clinical trial by integrating clinical pathways and EMRs. We report herein on a pilot test of our model in a single-centre clinical trial conducted at our institution.

Methods

Methods and outline of our model of the clinical trial process

We modelled a new clinical trial process in which practical medical records are used directly in clinical trials and developed a clinical trial system to follow our model. We applied the new system to an investigator-initiated clinical trial and examined whether all the data needed to conduct the clinical trial were extracted correctly throughout the system. Moreover, by comparing the new model with the conventional method of conducting clinical trials, we made a qualitative observation of how the new model reduces the workload in clinical trials.

In our model, a clinical trial is performed as follows (see also Figure 1):

1) In the protocol development stage, the methods of measuring endpoints (i.e., protocol treatment and evaluation items) are decided based on the clinical pathways that usually are used for patients with the same diagnosis. Next, medical record descriptions are defined, including eligibility criteria, tests, protocol treatment, efficacy of treatment, adverse events and follow-up. At the same time, the data format and coding conventions for the statistical data are defined to be compatible with statistical software. For example, the description of adverse events is synchronised and standardised with clinical practice and clinical trials according to the Common Terminology Criteria for Adverse Events (CTCAE) [16].
2) According to the defined descriptions, screens are prepared for entry of the additional data required for the clinical trial into EMRs using EMR templates and screens are prepared for data checks and data extraction by our EMR retrieval system (ERS). The templates are named to enable clinicians to identify them as pertaining only to the trial.
3) Patients who provide informed consent and agree to participate are registered and randomly assigned to one of the protocol treatments.
4) The protocol treatment assigned to the patient is executed according to clinical pathways, and the treatment data are recorded to EMRs using EMR templates.
5) Data are extracted using the ERS and are checked automatically for completeness and consistency and to identify anomalies to be resolved.
6) After edit checks have been satisfied following corrections, the data are extracted by the ERS, archived in portable document format (PDF) with an electronic signature, and transferred in comma-separated values (CSV) format to a coordinating centre.
7) At the coordinating centre, the clinical trial data from participating sites are checked, integrated, and made available for statistical analysis.

When it is necessary to update trial data that already have been transferred to the coordinating centre, the process repeats from when the investigator updates the medical records using the clinical trials template. In addition, central pathology reviews or central laboratory findings are integrated with clinical data in the coordinating centre.

Because our new model is integrated, with the routine documentation of clinical practice and the procedures of clinical trials, to avoid redundant data entries, the efficiency of clinical trials can be improved.

Applied information technology

EMR templates

To record clinical trial data to EMRs that could be available for clinical practice, we used EMR template technology [14,17–21]. In the current EMR system in Japan, various types of information are integrated, including coded data. Coded data include laboratory results that are in the computerised physician order entry system, narrative information, such as observations during physical examinations or progress notes that are written in free-text form, radiological images, and waveform information from electrocardiograms. In particular, it was necessary to convert free-text information to structured information that is expressed by data elements consisting of items and values with codes for use in a clinical trial [17]. The most practical method is to use a template for entering data into the EMR system [17]. Using templates, the entered narrative information can be made available for data analysis.

In Japan, the EMR template technology already has been implemented for most large hospitals' EMR systems, including ours, and is widely used to standardise medical records. Although multiple vendors supply various templates, when the new information is recorded, our hospital stores key data, such as the template ID, item ID, and entry date and time, in the EMR database. Moreover, our EMR system is managed according to the Japanese

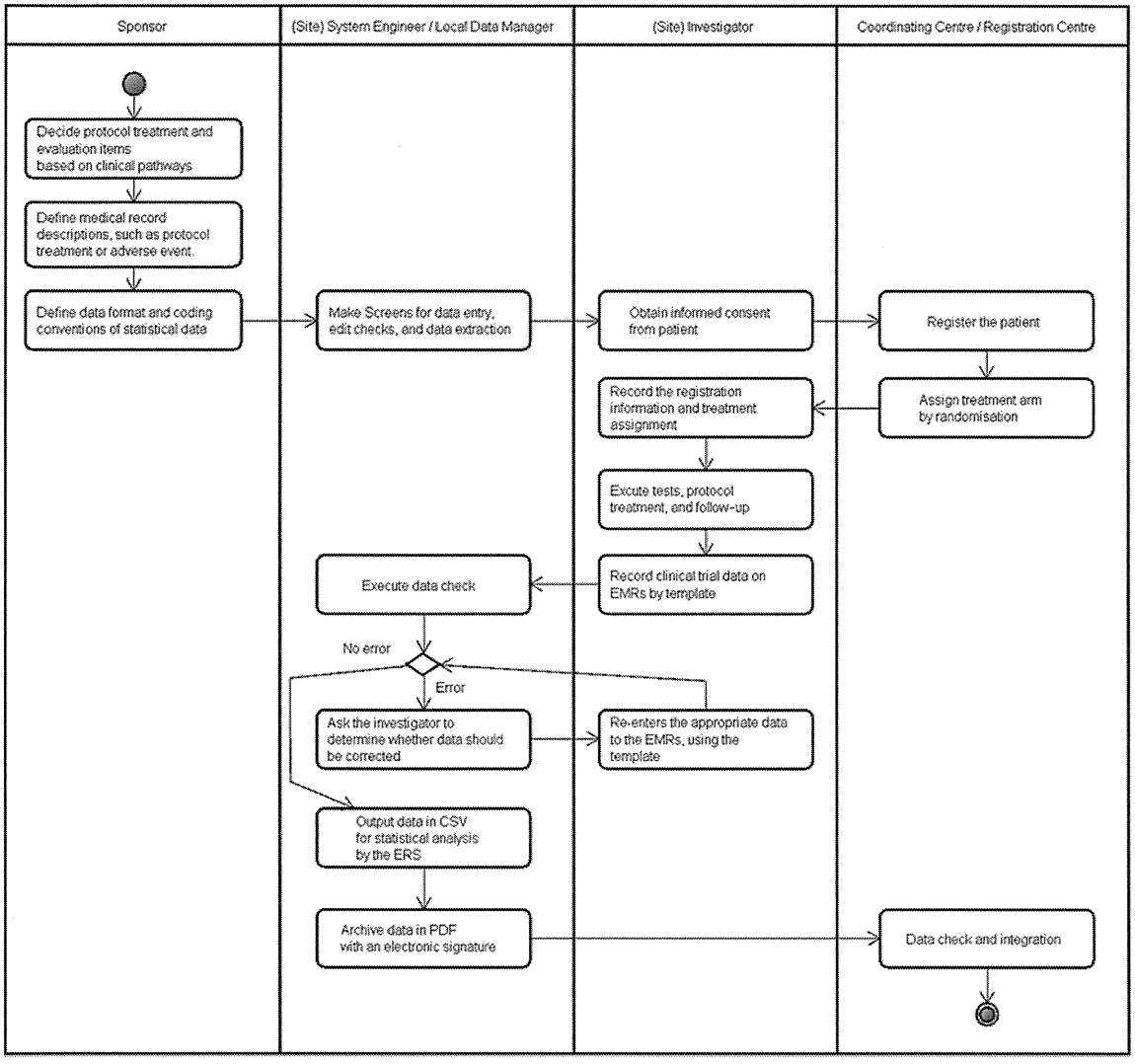

Figure 1. Our new model of the clinical trial process.
CSV: comma-separated values; ERS: EMR retrieval system; PDF: portable document format; EMRs: electronic medical records.

Ministry of Health, Labour and Welfare's guidelines on hospital information system management. To guarantee data integrity, all modifications to EMR data are recorded when and by whom each operation is executed, and an audit trail is made automatically. Audit trails record whose, when, and by whom medical records have been accessed. No user can update audit trails or prevent recording them. In addition, after medical records are submitted by medical staff, no user can delete the records. When medical records are updated, the new data are created as updated records, and the old data are retained as deleted records with a 'deletion flag'. This traceability management function is applied to the clinical trial template; all clinical trial data and histories are stored on the EMR system.

EMR retrieval system

We use the ERS that was developed in our translational research centre to extract data from EMRs, verify the completeness and consistency of trial data, and create output data for statistical analysis. The ERS can retrieve data from patients participating in the clinical trial comprehensively and efficiently. To retrieve and report the records in clinical practice directly as data for clinical research, we identified entities from EMRs that are useful for clinical research (i.e., data on patient demographics, diagnosis, physical examination, progress notes recorded in the EMR template, operative notes, laboratory tests, radiological or pathological studies, medications and injections, and other treatments). Next, we designed a multidimensional data model