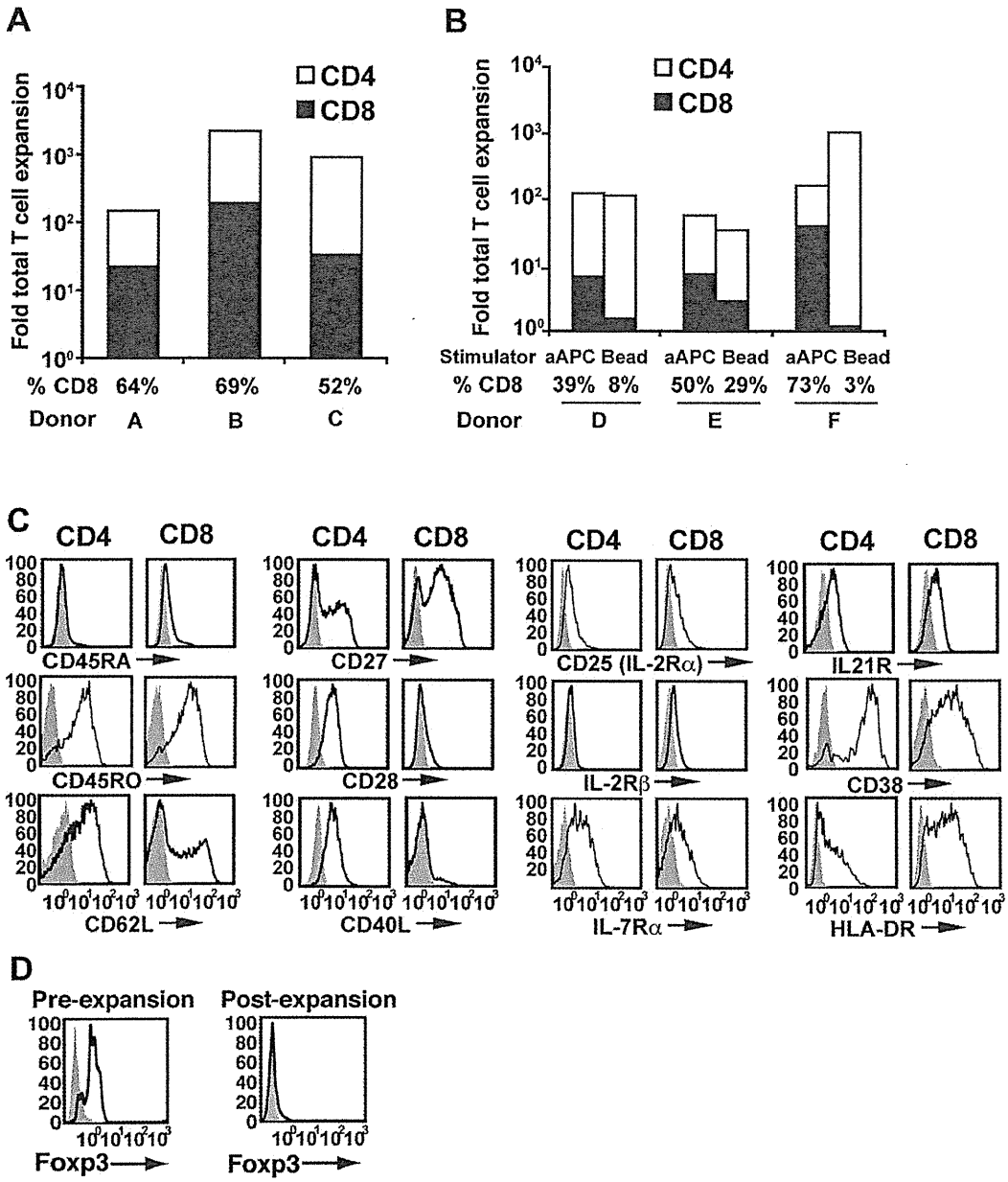

Figure 2. aAPC/mOKT3 expands both CD4+ and CD8+ T cells without using allogeneic feeder PBMC. (**A**) CD3+ T cells were stimulated twice with aAPC/mOKT3 and supplemented with IL-2 between stimulations. Fold expansion of CD3+ T cells over one month is shown for three donors. Shading shows the proportion of expanded CD4+ (white) and CD8+ (black) T cells, and percent CD8+ T cells is indicated. (**B**) CD3+ T cells were stimulated twice with aAPC/mOKT3 or beads (Dynabeads CD3/CD28) and supplemented with IL-2 between stimulations. Fold expansion of CD3+ T cells over one month is shown for three donors. Shading shows the proportion of expanded CD4+ (white) and CD8+ (black) T cells, and percent CD8+ T cells is indicated. (**C**) CD3+ T cells were expanded as described in Figure 2A. Expression of surface molecules on gated CD4+ and CD8+ T cells is shown (open). Isotype mAb staining was used as a control (shaded). (**D**) CD4+ CD25+ Foxp3+ Treg cells, present pre-expansion, were absent in expanded cultures. CD4+ CD25+ cells, pre- and post-expansion, were stained intracellularly with anti-Foxp3 mAb (open) and isotype control (shaded).
doi:10.1371/journal.pone.0030229.g002

aAPC/mOKT3 expands functional TIL but not contaminating Treg cells

Using aAPC/mOKT3, lymphocytes derived from malignant ascites (breast and ovarian cancer) and melanoma metastases were successfully expanded without adding any allogeneic feeder cells (Figure 4A). As observed with peripheral CD3+ T cells in Figure 2A, CD8+ T cells predominantly expanded in all cultures, including those that initially contained a minimal percentage of CD8+ T cells. Importantly, Foxp3+ cells did not proliferate well (Figure 4B). As with peripheral CD3+ T cells, expanded TIL had a central memory~effector memory phenotype (CD45RA- CD62L+/-) consistent with a lack of terminal differentiation (Figure S2). Furthermore, expanded T cells highly expressed CD27 and CD28 which are associated

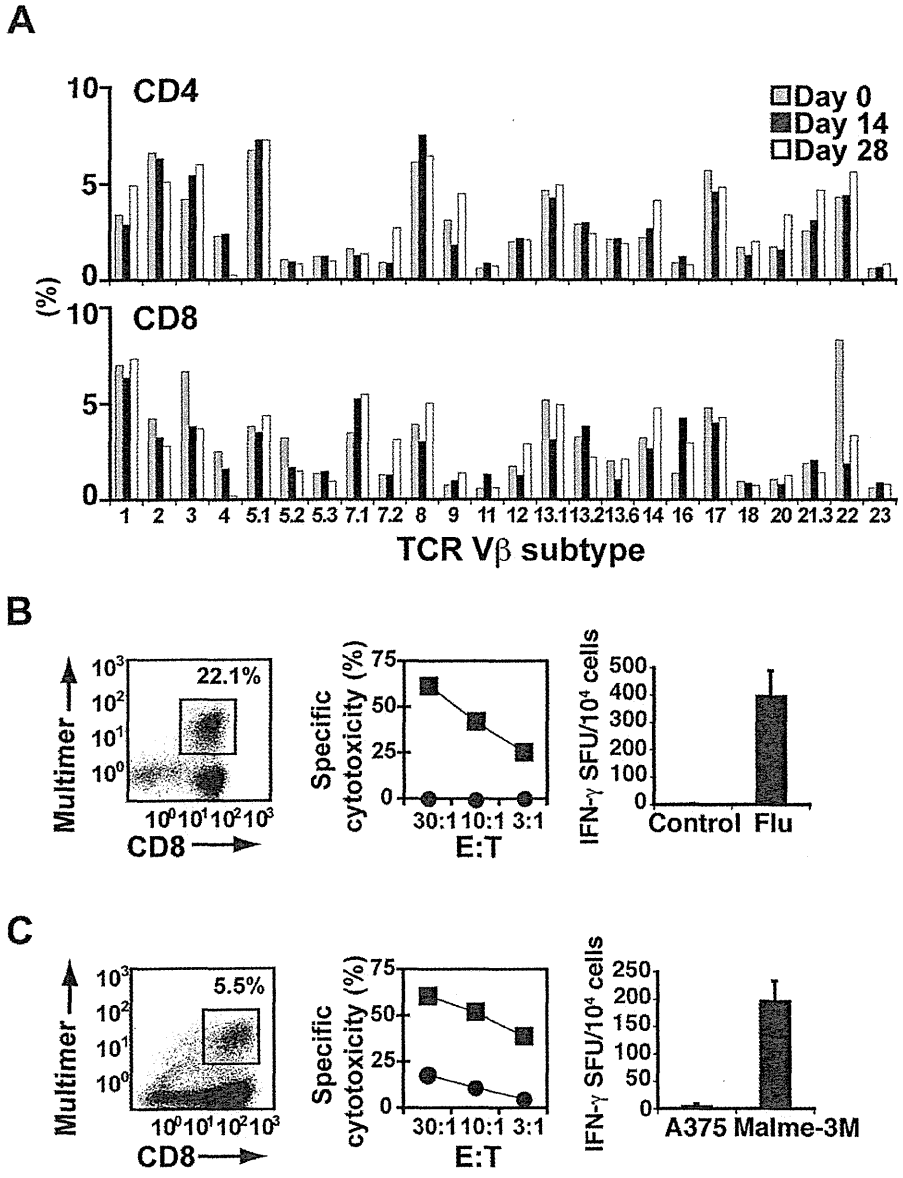

Figure 3. Expansion with aAPC/mOKT3 does not induce skewing of the TCR Vβ repertoire. (**A**) TCR Vβ subfamily analysis before and after stimulation with aAPC/mOKT3 is shown. $CD3^+$ T cells were stimulated with aAPC/mOKT3 on days 0 and 14 and were treated with IL-2 at 300 IU/ml between stimulations. TCR Vβ usage analysis was performed on days 0, 14, 28. Data shown is on gated $CD4^+$ and $CD8^+$ T cells. (**B, C**) $A2^+$ $CD3^+$ T cells were stimulated twice with aAPC/mOKT3 for one month. Subsequently, $CD8^+$ T cells were purified from expanded $CD3^+$ T cells and further stimulated with aAPC/A2 pulsed with Flu or MART1 peptide. (**B**) Flu specificity was demonstrated by multimer staining (left). Functional competence was demonstrated by antigen-specific cytotoxicity (middle) and IFN-γ secretion (right). T2 cells pulsed with Flu peptide (■) or control peptide (●) were used as targets. (**C**) MART1 specificity was similarly demonstrated by multimer staining (left). The HLA-$A2^+$/$MART1^+$ melanoma line, Malme-3M (■), and the HLA-$A2^+$/$MART1^-$ melanoma line, A375 (●), were used as targets in cytotoxicity (middle) and IFN-γ ELISPOT assays (right).
doi:10.1371/journal.pone.0030229.g003

with T cell survival and persistence *in vivo* [56-59]. They also secreted high quantities of IFN-γ and IL-2, while IL-4 secretion was lower and no IL-10 was produced (Figure 4C). These results demonstrate that the aAPC/mOKT3-based system can expand tumor-infiltrating $CD8^+$ T cells in the presence of autologous $CD4^+$ T cells, and that they display phenotypic and functional characteristics consistent with central memory~effector memory T cells.

IL-2 and IL-21 are necessary, but not sufficient, for $CD4^+$ T cell-mediated help of $CD8^+$ T cell expansion

Using the aAPC/mOKT3-based expansion system, we compared the expansion of $CD8^+$ T cells in the presence or absence of $CD4^+$ T cells. $CD8^+$ T cells expanded much better in the presence of $CD4^+$ T cells (Figure 5A), suggesting the presence of $CD4^+$ T cell help for $CD8^+$ T cells in these aAPC/mOKT3-based cultures. We tested whether this "help" was mediated by soluble factors or

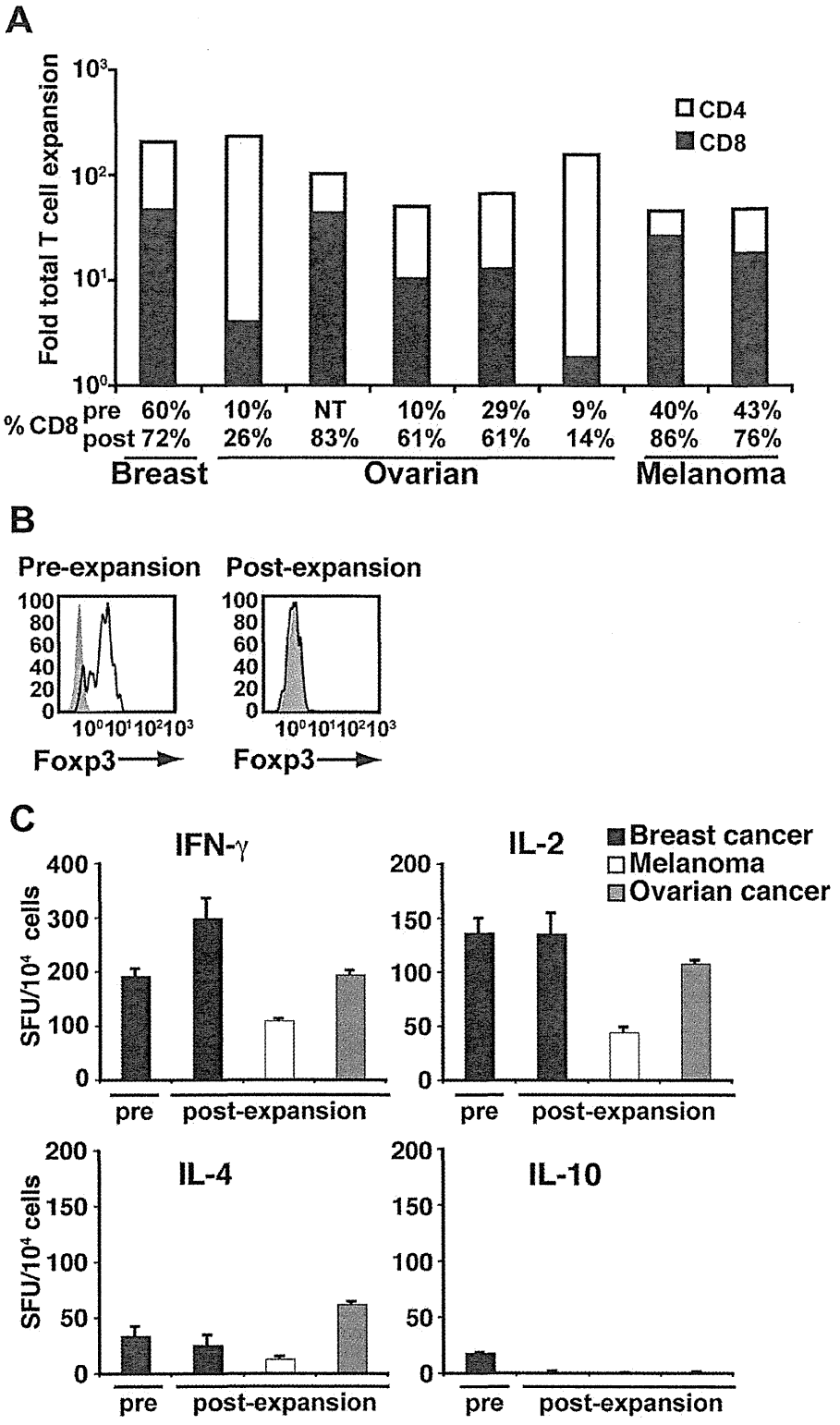

Figure 4. aAPC/mOKT3 expanded TIL are Foxp3 negative and secrete predominantly Th1 cytokines. (**A**) Expansion of TIL obtained from breast and ovarian cancer ascites and melanoma metastases is shown. Shading indicates the proportion of $CD4^+$ (white) and $CD8^+$ (black) T cells in expanded cultures. The percentage of $CD8^+$ T cells in pre- and post-expansion cultures is shown. Note that in all samples tested, the percentage of $CD8^+$ T cells increased even in those that initially contained a minimal percentage of $CD8^+$ T cells. NT denotes not tested. (**B**) $CD4^+$ $CD25^+$ $Foxp3^+$ Treg

cells, present pre-expansion, were not detectable after one month of culture. CD4+ CD25+ cells were intracellularly stained with anti-Foxp3 mAb (open) and isotype control (shaded). (**C**) IFN-γ, IL-2, IL-4, and IL-10 secretion of expanded TIL was determined by ELISPOT assays. Cytokine secretion by TIL from the breast cancer ascites specimen prior to expansion is shown as a control. Pre-expansion samples from melanoma and ovarian cancer specimens were not studied because of low initial cell numbers.
doi:10.1371/journal.pone.0030229.g004

cell-cell contact using the transwell assay (Figure 5B). A single stimulation, without any exogenously added cytokines, expanded CD8+ T cells by an average of 40.5% better when CD4+ T cells were present but separated from CD8+ T cells by the transwell membrane ($P<0.005$). In co-cultures where CD4+ and CD8+ T cells were mixed, allowing for direct cell-cell contact, CD8+ T cells expanded more than in cultures where they were separated from CD4+ T cells by the transwell membrane ($P<0.05$). These results suggest that observed CD4+ T cell help involves both soluble factors and cell-cell contact.

To identify molecules mediating the observed CD4+ T cell help, culture supernatants of CD4+/CD8+ T cell mixed and separate cultures were tested for a panel of soluble factors (Figure 5C and Table S1). Greater quantities of MIP-1α, MIP-1β, and RANTES were detected in CD4+/CD8+ T cell mixed cultures compared to separate cultures, suggesting increased production in mixed cultures. In contrast, IL-2 and IL-21, as well as IL-10, IL-17, TNF-α, and TNF-β, were detected at lower levels in mixed cultures, consistent with more consumption or less production of these cytokines.

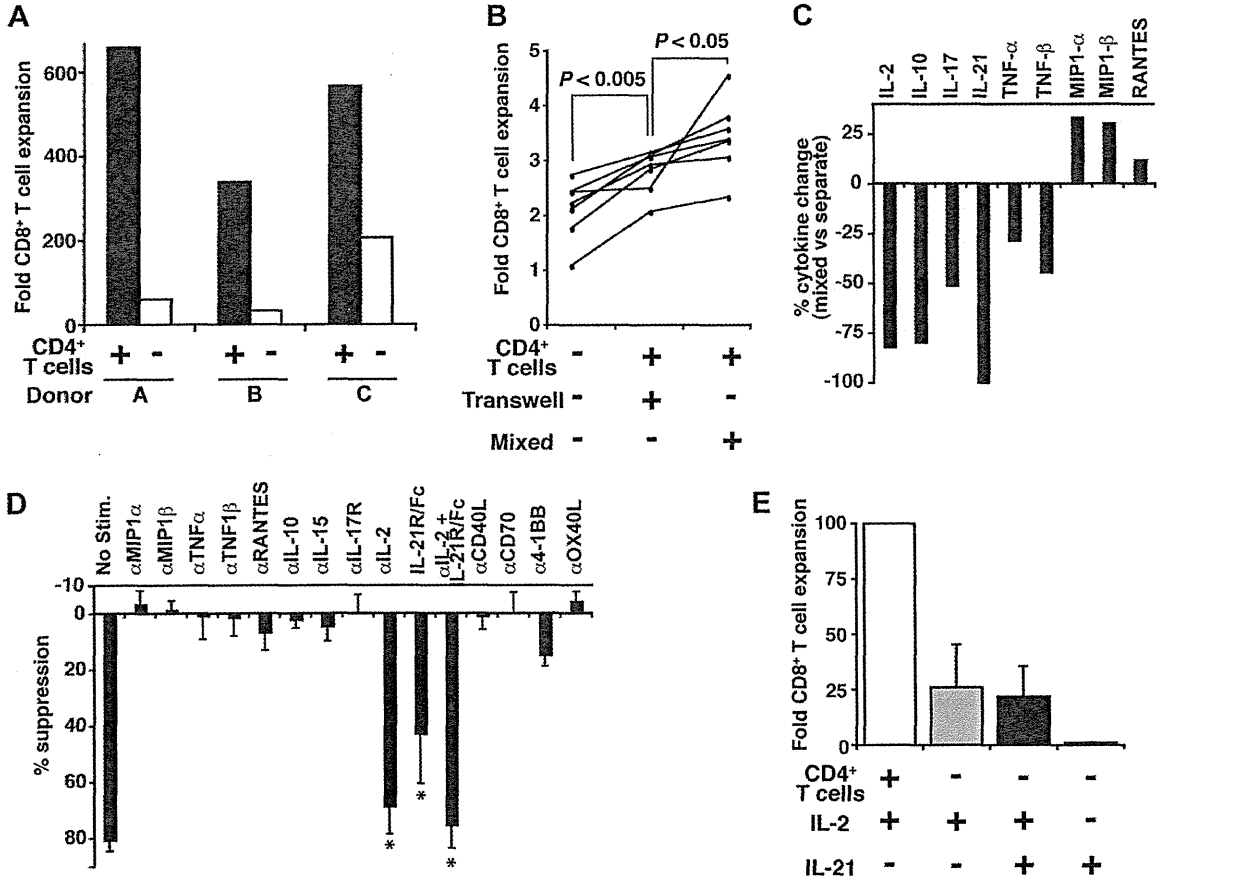

Figure 5. Autologous CD4+ T cell secretion of IL-2/IL-21 is necessary but not sufficient to help CD8+ T cells proliferate. (**A**) CD8+ T cells were stimulated twice by aAPC/mOKT3 with or without CD4+ T cells and treated with IL-2 between stimulations. Fold expansion of CD8+ T cells over 28 days is shown for 3 donors. (**B**) CD8+ T cells were stimulated only once by aAPC/mOKT3 with or without CD4+ T cells in transwell plates. No IL-2 or other cytokines were given. Fold expansion of CD8+ T cells over 6 days is shown for 7 donors. (**C**) Culture supernatants were tested for a panel of soluble factors to identify mediators of CD4+ T cell help. Relative changes in cytokines, comparing mixed vs. separate cultures, are shown. Data is representative of two donors. Absolute values for two donors are shown in Table S1. (**D**) Suppression of CD8+ T cell expansion in the presence of CD4+ T cells by blocking reagents is presented as percent suppression relative to control. Values indicate mean of four independent experiments; error bars show s.d. *$P<0.005$. (**E**) CD8+ T cells were stimulated twice with aAPC/mOKT3 in the presence or absence of CD4+ T cells. IL-2, IL-21, or both were added in each condition. Fold expansion of CD8+ T cells over 28 days is shown. Percent expansion was calculated by dividing the number of expanded CD8+ T cells by the number of CD8+ T cells expanded in the presence of CD4+ T cells. Values indicate mean of six independent experiments; error bars show s.d.
doi:10.1371/journal.pone.0030229.g005

To differentiate between "more consumption" and "less production," $CD4^+/CD8^+$ T cell mixed cultures were stimulated in the presence of blocking reagents, and suppression of $CD8^+$ T cell expansion was assessed (Figure 5D). Blockade of IL-2 and IL-21 resulted in a reduction of expansion by 68.8% ($P<0.005$) and 42.9% ($P<0.005$), respectively. These results indicate that the decreased levels of IL-2 and IL-21 in $CD4^+/CD8^+$ T cell mixed cultures were due to more consumption rather than less production and that these cytokines may be necessary mediators of $CD4^+$ T cell help in this human-based in vitro system. To test whether IL-2/IL-21 could substitute for the observed $CD4^+$ T cell help, $CD8^+$ T cells stimulated with aAPC/mOKT3 were supplemented with IL-2, IL-21, or both (Figure 5E). $CD8^+$ T cells did not expand without IL-2. The addition of IL-2 with or without IL-21 did not improve $CD8^+$ T cell expansion to the level observed when cocultured with $CD4^+$ T cells, demonstrating that IL-2 plus IL-21 are not sufficient to replace $CD4^+$ T cell help.

Exogenous IL-2/IL-21 and upregulation of IL-21 receptor can partially recapitulate $CD4^+$ T cell help of $CD8^+$ T cell expansion *in vitro*

Interestingly, we observed that higher expression of the IL-21 receptor (IL-21R) on $CD8^+$ T cells occurred when $CD4^+$ T cells were present during stimulation by aAPC/mOKT3 (Figure 6A). Higher IL-21R expression on $CD8^+$ T cells was not induced by supplementing cultures with IL-2 and IL-21 (data not shown). This prompted us to hypothesize that increased upregulation of IL-21R on $CD8^+$ T cells is critical for the full effect of IL-21 secreted by $CD4^+$ T cells. We constitutively expressed IL-21R on $CD8^+$ T cells (Figure 6B, left) and stimulated them with aAPC/mOKT3 in the presence of IL-2/IL-21. In accordance with the transduction efficiency of IL-21R to 75.9%, $CD8^+$ T cell proliferation partially increased to levels seen in the presence of $CD4^+$ T cells (Figure 6B, right). This indicates that elevated expression of IL-21R is necessary and can partially recapitulate $CD4^+$ T cell help for $CD8^+$ T cell proliferation.

Discussion

A novel human cell-based aAPC expanded $CD3^+$ T cells *in vitro* without the addition of allogeneic feeder PBMC. Phenotypic analysis of expanded healthy donor T cells and TIL showed, that while both $CD4^+$ and $CD8^+$ T cells expanded, $CD8^+$ T cells predominated. In this model system, we demonstrated that $CD8^+$ T cell expansion depended on the presence of $CD4^+$ T cells, suggesting that $CD4^+$ T cells provided help to proliferating $CD8^+$ T cells. The $CD4^+$ T cell secreted cytokines, IL-2 and IL-21, and the $CD4^+$ T cell-dependent upregulation of IL-21R on $CD8^+$ T cells were necessary for the observed $CD4^+$ T cell help.

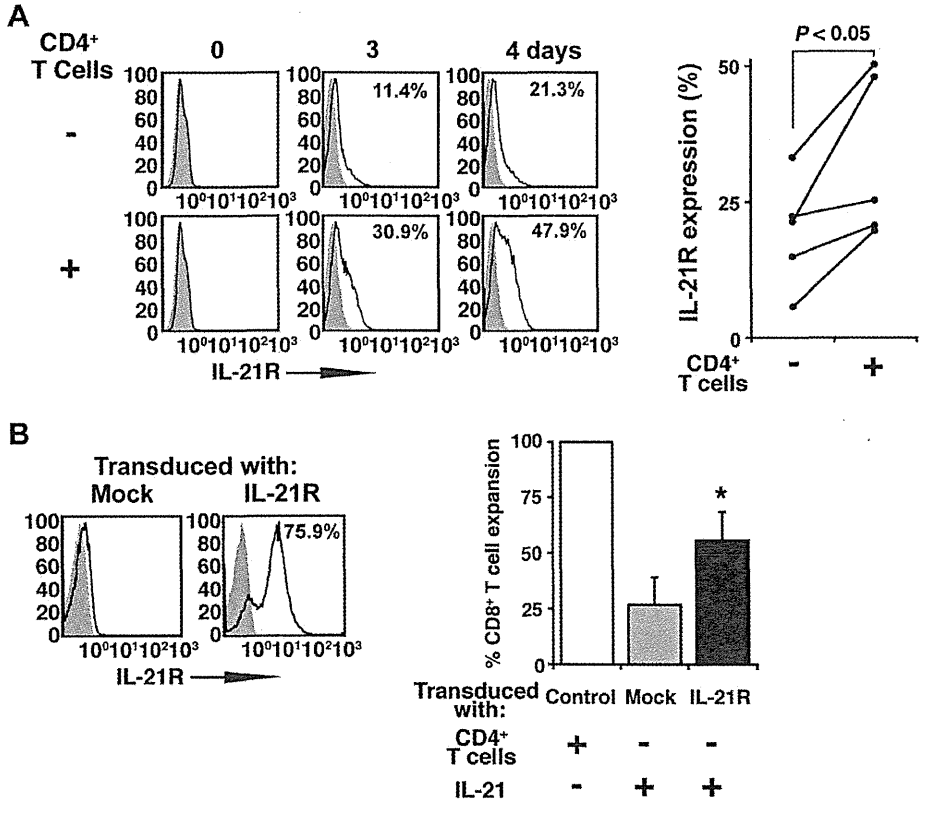

Figure 6. IL-2/IL-21 and upregulation of IL-21R expression replace $CD4^+$ T cell help of $CD8^+$ T cell expansion *in vitro*. (**A**) IL-21R expression on $CD8^+$ T cells stimulated with aAPC/mOKT3 in the presence or absence of $CD4^+$ T cells was studied by flow cytometry. On the left, histogram plots for 1 donor is shown and, on the right, IL-21R expression on day 4 is displayed for 5 donors. (**B**) IL-21R expression on $CD8^+$ T cells ectopically transduced with mock or IL-21R is shown (left). Expansion of transduced $CD8^+$ T cells stimulated twice by aAPC/mOKT3 with or without IL-21 is compared (right). Percent expansion was calculated by dividing the number of expanded transduced $CD8^+$ T cells by that of $CD8^+$ T cells stimulated in the presence of $CD4^+$ T cells. Values indicate mean of four independent experiments; error bars show s.d. *$P<0.005$.
doi:10.1371/journal.pone.0030229.g006

IL-2 and IL-21 have previously been shown to mediate $CD4^+$ T cell help in murine *in vivo* studies. IL-2, one of the few effector cytokines made by naïve $CD4^+$ T cells, expands activated T cells and is essential in the development of $CD8^+$ T cell memory responses to pathogens [60]. While $CD8^+$ T cell responses during acute viral infections were relatively independent of IL-2, the development of protective $CD8^+$ T cell memory responses required IL-2 exposure during priming [35–37]. In vivo models also indicate that IL-21 is critical for containing chronic viral infections and preventing the deletion of high affinity antiviral $CD8^+$ T cells. IL-21 secretion by $CD4^+$ T cells enables the generation, sustained proliferation, and maintenance of polyfunctional $CD8^+$ T cells during chronic infection [39–41].

Our results confirmed a role for IL-2 and IL-21 in human $CD4^+$ T cell help. By using a standardized aAPC, we were able to single out and examine the effects of cocultured $CD4^+$ T cells, unhindered by immunostimulatory and inhibitory factors produced by allogeneic feeder cells. Stimulation of T cells with aAPC/mOKT3 induced the secretion of cytokines and chemokines, including high levels of interferon-γ, MIP-1α, and MIP-1β. Among all the cytokines and chemokines studied, blocking experiments identified IL-2 and IL-21 as necessary for $CD4^+$ T cell help of $CD8^+$ T cell expansion. These cytokines alone, however, were not sufficient to replace $CD4^+$ T cells. We showed that $CD4^+$ T cells help by enhancing IL-21R expression on $CD8^+$ T cells, rendering them more responsive to secreted IL-21. Taken together, the secretion of IL-2/IL-21 and the induction of IL-21R are necessary and sufficient to partially recapitulate human $CD4^+$ T cell help of $CD8^+$ T cell expansion *in vitro*.

Transwell assays showed that the $CD4^+$ T cell dependent expansion of $CD8^+$ T cells was also mediated by cell-cell contact factors. CD40-CD40 ligand interactions have been shown to mediate $CD4^+$ T cell help through CD40-mediated activation of dendritic cells, which are then "licensed" to stimulate $CD8^+$ T cells [43,44,61]. CD40 ligation was also shown to increase IL-21R expression on B lymphocytes suggesting a mechanism for IL-21R upregulation on $CD8^+$ T cells [62]. However, we did not observe any suppression of $CD8^+$ T cell expansion following blockade of CD40 ligand (Figure 5D) even though expanded $CD4^+$ T cells strongly expressed CD40 ligand (Figure 2C). Furthermore, stimulation with aAPC/mOKT3 in the presence of CD40 ligation and the addition of IL-21 did not consistently enhance $CD8^+$ T cell expansion (data not shown). Therefore, these results are in agreement with others who have shown that $CD4^+$ T cells do not provide direct help to $CD8^+$ T cells through CD40 ligation [63,64]. It should be noted that blocking of CD70, 4-1BB, or OX40 signaling also did not suppress the expansion of $CD8^+$ T cells in the presence of $CD4^+$ T cells (Figure 5D).

aAPC induced polyclonal expansion of both $CD4^+$ and $CD8^+$ T cells as shown by the absence of clonal skewing of the TCR Vβ repertoire. The ability to further expand antigen-specific T cells capable of killing tumor targets indicated that the TCR repertoire for highly avid T cells was preserved. Also, expanded TIL secreted higher amounts of Th1 cytokines, IFN-γ and IL-2, which are associated with anti-tumor immunity. While aAPC/mOKT3 induced substantial expansion of $CD8^+$ T cells in the presence of $CD4^+$ T cell help, terminal effector T cell differentiation did not occur, as demonstrated by the central memory~effector memory phenotype ($CD45RA^-$ $CD45RO^+$ $CD62L^{+/-}$). Retention of CD62L expression would enable homing to lymph nodes, where encounter with antigen presented by professional APC could augment immune responses [65]. CD27, which is down-regulated in late stage effector T cells, was also highly expressed. CD27 expression by *in vitro* expanded TIL and T cell clones has been associated with persistence and clinical responses after adoptive transfer [56,57,59,66].

We also found that expanded T cells were not contaminated by cells with the $CD4^+$ $CD25^+$ $Foxp3^+$ Treg phenotype even when $CD4^+$ $CD25^+$ $Foxp3^+$ T cells were present prior to stimulation. We previously found that K562-based aAPC expressing HLA-DR molecules did not expand $Foxp3^+$ cells even though aAPC itself produces modest amounts of the Treg cell growth factor TGF-β [48]. We previously reported that aAPC also secretes IL-6 [47]. It is possible that IL-6, secreted by aAPC, might interfere with $Foxp3^+$ Treg cell expansion [67,68].

Adoptive transfer of *in vitro* expanded T cells has led to clinically significant anti-tumor responses in patients [30]. By leveraging autologous $CD4^+$ T cell help, aAPC/mOKT3 eliminates the use allogeneic feeder cells for T cell expansion, potentially increasing the availability of adoptive therapy as a cancer treatment. We previously reported the development of K562-based aAPCs dedicated to the expansion of HLA-restricted antigen-specific $CD4^+$ and $CD8^+$ T cells [47,48]. Antigen-specific $CD4^+$ and $CD8^+$ T cells expanded *in vitro* with these aAPC had a central memory~effector memory phenotype ($CD45RA^-$ $CD62L^{+/-}$) and possessed surprisingly prolonged *in vitro* longevity without feeder cells or cloning. In a recent clinical trial, HLA-A2-restricted MART1 peptide-specific $CD8^+$ T cells generated *in vitro* with aAPC were infused to advanced melanoma patients [69]. Without lymphodepletion or IL-2 administration, transferred T cells could persist for >16 months, established anti-tumor immunological memory *in vivo*, trafficked to tumor, and induced clinical responses. aAPC/mOKT3 extends the K562 platform to the stimulation of T cells regardless of HLA subtype. The aAPC/mOKT3-based T cell expansion system facilitates the understanding of mechanisms for human $CD4^+$ T cell help and provides a novel strategy to expand T cells for *in vitro* and *in vivo* uses.

Materials and Methods

Ethics Statement

All specimens and clinical data were collected under protocols approved by the Institutional Review Board at the Dana-Farber Cancer Institute (DFCI). All patients provided written informed consent for the collection of samples and subsequent analysis.

cDNAs and cell lines

cDNAs encoding the heavy and light chains for a membranous form of anti-CD3 mAb (OKT3, mIgG2a) were cloned from hybridoma cells (ATCC, VA). HLA null K562 transduced with CD80 and CD83 has been described previously [47,53]. $CD80^+$ $CD83^+$ K562 cells were retrovirally transduced with the heavy and light chains of a membranous form of anti-CD3 mAb. After drug selection, anti-CD3 mAb expressing cells were isolated by magnetic bead guided sorting (Miltenyi Biotec, CA). High expression of a membranous form of anti-CD3 mAb on the cell surface was confirmed by flow cytometry. The parental cell line K562 lacks the endogenous expression of any HLA molecule, but does endogenously express the adhesion molecules CD54 and CD58.

Retrovirus supernatants expressing IL-21R was harvested from PG13 cells. Fresh $CD8^+$ T cells purified from healthy donors were first activated with anti-CD3 (0.75 μg/ml) and anti-CD28 (1 μg/ml) mAbs (Fitzgerald Industries International, MA) for two days. Pre-activated T cells were infected with IL-21R or mock retrovirus supernatants every 24 hr at an MOI of 10 for 10 days and treated with 50 IU/ml IL-2 between infections. Following the assessment

of IL-21R expression by flow cytometry analysis, infected T cells were stimulated with aAPC/mOKT3.

T2, A375, and Malme-3M cell lines were obtained from ATCC as described elsewhere [47].

T cell expansion

Healthy donor PBMC were obtained by leukapheresis performed at the DFCI Kraft Family Blood Donor Center. Cells were isolated by Ficoll-Hypaque density gradient centrifugation and $CD3^+$, $CD4^+$, or $CD8^+$ T cells were purified by negative selection via MACS sorting according to the manufacturer's protocol (Miltenyi Biotec, CA). TIL samples were processed by centrifugation of malignant ascites or mechanical and enzymatic digestion of melanoma metastases with collagenase as previously described [70]. $CD3^+$ TIL were obtained by positive or negative selection via MACS sorting (Miltenyi Biotec, CA). aAPC/mOKT3 cells were irradiated (200 Gy) and added to purified T cells at a T cell to aAPC ratio of 20:1 unless otherwise noted. Dynabeads CD3/CD28 (Invitrogen, CA) were used as stimulators according to the manufacturer's instruction at a T cell to bead ratio of 1:3. Expanding T cells were cultured in RPMI 1640 containing 10% human AB sera and gentamycin (Invitrogen, CA), and between stimulations, unless otherwise noted, 300 IU/ml IL-2 (Prometheus, CA) was added every 3-4 days. In the absence of $CD4^+$ T cells, $CD8^+$ T cells expanded only in the presence of IL-2. Where indicated, 50 ng/ml IL-21 (Peprotech, NJ) was added every 3-4 days. Unless otherwise noted, T cells were restimulated every two weeks. Expanded cells were characterized two weeks after the second stimulation. Cell viability was >90% by trypan blue exclusion.

To test whether antigen-specific cultures can be generated from $CD3^+$ T cells polyclonally expanded with aAPC/mOKT3, $CD3^+$ T cells derived from HLA-A*0201 $(A2)^+$ donors were initially stimulated and expanded with aAPC/mOKT3 for one month. Subsequently, $CD8^+$ T cells were purified and further stimulated with Flu or MART1 peptide-pulsed aAPC/A2 as previously described [47,53].

Analysis of cultured T cells

Flow cytometry analysis was performed using mAbs for the following antigens: CD4, CD8, CD25, CD28, CD56, CD62L, and IL-2Rβ (Coulter, CA); CD40 ligand, CD80, IL-7Rα, OX40, OX40 ligand, and 4-1BB (BD Biosciences, CA); CD27, CD45RA, CD45RO and CD83 (Invitrogen, CA); CCR4 and CCR7 (R&D Systems, MN); ICOS, NKG2D, and PD-1 (eBioscience, CA); CD38, Foxp3, HLA-DR, and 4-1BB ligand (Biolegend, CA); CD40 and CD70 (Ancell, MN); IL-21R (R&D Systems, MN; or BD Biosciences, CA). Goat anti-mouse IgG (H+L) Fab (Jackson ImmunoResearch, PA) was used to detect surface expression of murine Ig. Assessment of TCR Vβ subfamily usage was performed using TCR Vβ mAbs (Beta Mark, Coulter, CA).

To assess the production/consumption of soluble factors in T cell cultures, purified $CD4^+$, $CD8^+$, or a 1:1 mixture of $CD4^+$ and $CD8^+$ T cells were stimulated with irradiated aAPC/mOKT3 for 72 hours and supernatants were measured for: GM-CSF, IFN-γ, IL-2, IL-4, IL-10, IL-12, IL-15, IL-17, MIP-1α, MIP-1β, RANTES, TNF-α, TNF-β, and TRAIL (R&D Systems, MN); IL-7 (Diaclone/Cell Sciences, MA); IL-18 (Medical & Biological Laboratories, Japan); and IFN-α (PBL Biomedical Laboratories, NJ). IL-21 (eBiosciences, CA) was measured at 48-hours. Relative changes in cytokines resulting from mixed cultures of $CD4^+$ and $CD8^+$ T cells vs. separate $CD4^+$ and $CD8^+$ T cell cultures were determined by the following formula: (x-y)/y, where x = cytokine secreted by $CD4^+$ and $CD8^+$ T cell mixed co-cultures and y is the average of cytokine produced in separately stimulated $CD4^+$ and $CD8^+$ T cell cultures.

IFN-γ ELISPOT and standard chromium release assays were performed as described elsewhere [47,53]. IL-2, IL-4 and IL-10 ELISPOT assays were performed according to the manufacturer's protocol (R&D Systems, MN).

Transwell and blocking assays

Transwell assays were performed by placing purified $CD4^+$, $CD8^+$, or a mixture of $CD4^+$ and $CD8^+$ T cells into Millicell-24 plate chambers (Millipore) which were separated by a 0.4 μm filter allowing free movement of soluble factors but not cells. T cells were stimulated once with aAPC/mOKT3 in the absence of exogenous cytokines. Six days later, expansion of $CD8^+$ T cells was determined.

Blocking assays were performed in 96-well round bottomed plates where $CD4^+$ and $CD8^+$ T cells were combined 1:1 and then stimulated with irradiated mOKT3/aAPC in the presence of blocking reagents. Blocking mAbs used recognized IL-2, IL-10, IL-15, IL-17R, MIP-1α, MIP-1β, OX40 ligand, RANTES, TNFα, and TNFβ (R&D Systems, MN); 4-1BB (Neomarkers, CA); CD40 ligand (Biolegend, CA); and CD70 (Ancell, MN). IL-21 was blocked using recombinant human IL-21R subunit/Fc chimeric protein (R&D Systems, MN) as previously described [71]. Six days later, $CD8^+$ T cell expansion was determined.

Statistical analysis

Data analysis was performed using the paired, one-sided Student's t-test where $P<0.05$ was considered to be statistically significant.

Supporting Information

Figure S1 K562-based aAPC/mOKT3, expressing a membranous form of anti-CD3 mAb, stimulates $CD3^+$ T cell expansion. **(A)** $CD3^+$ T cells were stimulated twice with aAPC/mOKT3 and supplemented with IL-2 at the following concentrations: 10 IU/ml (gray), 300 IU/ml (white) and 6,000 IU/ml (black). Fold expansion over 28 days is demonstrated. Without IL-2 addition, T cell expansion over the 28-day culture period was minimal. Data for three separate donors is shown. **(B)** $CD3^+$ T cells were stimulated twice with aAPC/mOKT3 at the indicated aAPC: T cell ratios. Cultures were supplemented with IL-2 (300 IU/ml) between stimulations. Fold expansion of $CD3^+$ T cells over one month is shown for two donors. **(C)** Phenotype of fresh healthy donor $CD3^+$ T cells prior to stimulation is depicted to compare with the T cells shown in Figure 2C which were expanded with aAPC/mOKT3. Expression of surface molecules on gated $CD4^+$ and $CD8^+$ T cells is shown (open). Isotype mAb staining was used as a control (shaded). **(D)** HLA-$A2^+$ healthy donor $CD8^+$ T cells were stimulated with MART1 peptide-pulsed aAPC/A2 as previously described [47,53]. MART1 specific T cells were then stimulated twice with aAPC/mOKT3 in the presence of autologous $CD4^+$ T cells. Fold expansion of MART1 T cells over one month is shown for three donors.
(TIF)

Figure S2 TIL expanded with aAPC/mOKT3 express CD27 and CD28 and have a central memory~effector memory phenotype. $CD3^+$ T cells from malignant ovarian ascites were stimulated twice with aAPC/mOKT3, and cultures were supplemented with IL-2 at 300 IU/ml. **(A)** Fresh, unstimulated TIL and **(B)** aAPC/mOKT3 expanded TIL were stained with indicated mAb (open) and isotype control (shaded).

TIL were analyzed after a one month expansion. Data depicted is on gated $CD4^+$ and $CD8^+$ T cells.
(TIF)

Table S1 Soluble factors in T cell cultures stimulated with aAPC/mOKT3. Concentrations of soluble factors (pg/ml) in supernatants of $CD4^+$ separate, or $CD8^+$ separate, and $CD4^+$ and $CD8^+$ mixed T cell cultures stimulated by aAPC/mOKT3 were measured by ELISA. [a]Percent change was calculated as detailed in Methods. [b]not applicable. Data from two different donors is depicted.
(DOC)

Author Contributions

Conceived and designed the experiments: MOB LMN NH. Performed the experiments: MOB OI MT SA AB GM MIM MMM APM NH. Analyzed the data: MOB LMN NH. Contributed reagents/materials/analysis tools: MOB OI YY MT SA HM LMN NH. Wrote the paper: MOB LMN NH.

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ORIGINAL ARTICLE

An epidemiological study of the effects of statin use on airflow limitation in patients with chronic obstructive pulmonary disease

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ABSTRACT

Background and objective: **Chronic obstructive pulmonary disease (COPD) is considered to be a systemic inflammatory disease, and systemic inflammation has been noted as a factor contributing to cardiovascular disease, which is one of the comorbidities associated with COPD. On the other hand, pleiotropic effects, such as the anti-inflammatory effects of statins, have attracted attention in recent years, and there have been a variety of reports regarding the usefulness of statins for patients with COPD.**

Methods: **We investigated whether the use or non-use of statins influenced the prevalence of airflow limitation. All outpatients who were over the age of 40 years and who regularly visited a primary health care facility were invited to participate. Each participant underwent spirometry and completed a questionnaire regarding their clinical status, which was used to screen for COPD. A variety of factors that are potentially related to airflow limitation were assessed.**

Results: **Of the 853 patients included in the study, 81 (9.5%) had airflow limitation. The prevalence of airflow limitation was 2.3% among the 89 patients with a history of statin use, which was five times lower than the prevalence of airflow limitation among patients who had not used statins (10.5%). Among the 347 patients with a history of past or current smoking, airflow limitation was not observed in the 30 patients who had used statins. However, by multivariate analysis, statin use was not significantly associated with a lower prevalence of airflow limitation.**

Conclusions: **This is the first cross-sectional study from Japan that has demonstrated that statin use has a potential impact on airflow limitation in patients with COPD.**

SUMMARY AT A GLANCE

Statins have a variety of effects in patients with COPD. The prevalence of airflow limitation was approximately five times lower among patients using statins than among those not using statins. This is the first study from Japan demonstrating that statin use has a potential impact on airflow limitation.

Key words: airflow limitation, chronic obstructive pulmonary disease, cross-sectional study, statin use.

INTRODUCTION

Chronic obstructive pulmonary disease (COPD) is characterized by chronic airflow limitation and a range of pathological changes in the lungs, as well as significant extra-pulmonary effects and important comorbidities that may contribute to the severity of the disease.[1] The levels of inflammatory markers, such as serum C-reactive protein, tumour necrosis factor-α, interleukin-6 and interleukin-8, have been reported to be elevated in COPD patients,[2–5] and Young *et al.*[6] demonstrated the role of interleukin-6 in the pulmonary inflammation and matrix remodelling that underlies COPD. Recently, it has been suggested that COPD should be considered as a chronic systemic inflammatory disease.[7] In addition, it has been suggested that systemic inflammation in COPD may be strongly associated with cardiovascular disease, osteoporosis, weight loss and skeletal muscle atrophy.[1,6,8] With regard to the pathogenesis of COPD, the potential systemic spread of localized pulmonary inflammation is associated with smoking and other factors, and it is possible that pulmonary and systemic inflammation are induced by common risk factors such as current smoking;

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however, at present, the mechanisms associated with the pathogenesis of systemic inflammation in patients with COPD remain unknown.[9]

Statins play an important role in the prevention and treatment of hyperlipidaemia and atherosclerotic disease, and recently have been reported to have pleiotropic effects, including anti-inflammatory, anti-fibrotic and immunomodulatory effects.[10–12] Statins have also been reported to have a variety of effects in patients with COPD,[13–17] and it has been noted that they may represent a new treatment strategy for COPD.[6,18] Young *et al.*[6,19] suggested that through their anti-inflammatory effects, statins may confer a mortality benefit in patients with COPD and associated comorbidities, specifically coronary artery disease, chest infections and lung cancer. Although randomized controlled trials are needed to confirm and quantify these pulmonary effects, there is no doubt that statins reduce systemic inflammation and mortality, and are potentially useful in the management of COPD.[20]

In this study, we investigated whether the use or non-use of statins influenced the prevalence of airflow limitation. Each participant underwent spirometry and completed a questionnaire regarding their clinical status. A variety of factors that are potentially related to airflow limitation were assessed. Clinical parameters, including smoking status, current respiratory symptoms (cough, sputum, dyspnoea, wheeze) and use of statins for hyperlipidaemia and anti-hypertensive drugs for high blood pressure, were analysed.

METHODS

Subjects

This cross-sectional epidemiological study was conducted during the 1-year period from November 2007 to October 2008. All outpatients over the age of 40 years, who regularly visited 1 of 16 primary health care facilities affiliated with Jichi Medical University, were invited to participate in the study. Participants were recruited using poster advertisements or were orally invited to participate. Each participant underwent spirometry and completed a questionnaire regarding their clinical status, which was used to screen for COPD. A variety of factors that are potentially related to airflow limitation were analysed. The questionnaire was used to obtain information regarding the reason for the visit (treated disease), any respiratory disease that was previously diagnosed or treated, history of smoking, occupational history, other past medical history, the presence or absence of current respiratory symptoms (cough, sputum, dyspnoea, wheeze) and current use of medications (statins, angiotensin receptor blockers and angiotensin-converting enzyme inhibitors), and this information was subsequently confirmed by the primary physicians.

Spirometry was performed for those patients whose disease was stable, excluding those who had a previous history or treatment for COPD and asthma, which may cause chronic airway obstruction. For all 853 patients who gave written informed consent, spirometry was performed by the same clinical laboratory technologist, who was an expert in performing pulmonary function tests and who worked in each facility for 2–3 days. The pulmonary function tests were performed according to the procedures and criteria described in the Global Initiative for Chronic Obstructive Lung Disease.[1] The same spirometer (HI101, Chest, Inc., Tokyo, Japan) was used at all of the facilities. Each pulmonary function test was performed three times. Forced expiratory volume in 1 s (FEV_1)/forced vital capacity was calculated from the maximum FEV_1 and maximum forced vital capacity, and an FEV_1/forced vital capacity of <70% was taken as signifying the presence of airflow limitation. In addition, the severity of COPD was determined from the percentage FEV_1 relative to the predicted value (mild, ≥80%; moderate, ≥50% but <80%; severe, ≥30% but <50%; most severe <30%).[1] The institutional Ethics Committee at Jichi Medical University Hospital approved the study protocols and all participants gave written informed consent.

Statistical analyses

Statistical analysis of the relationship between airflow limitation and the demographic characteristics of the patients was performed using the Cochran–Armitage test or Fisher's exact test. The odds ratios for airflow limitation in patients who did or did not use statins were calculated, with adjustment for smoking history. The odds ratio homogeneity between smokers and non-smokers was determined by the Breslow–Day test. Logistic regression analysis was performed, with airflow limitation as the dependent variable, and age, gender, smoking status, pack-years of smoking, the number of current respiratory symptoms and use or non-use of statin as independent factors. Significance was set at $P < 0.05$. SAS version 9.1.3 software (SAS Institute Inc., Cary, NC, USA) was used for statistical analyses.

RESULTS

Spirometry was performed on 853 patients (416 males, 437 females, mean age 64.0 ± 11.3 years), who completed the questionnaire and did not have COPD. There were no incomplete or missing data for these 853 patients, and data for all patients was included in the analysis. Airflow limitation was detected in 81 (9.5%) of the 853 patients. Although the prevalence of airflow limitation differed at the 16 facilities participating in the study, there were patients with airflow limitation at all the participating facilities, regardless of the type of medical practice. The prevalence of airflow limitation according to age and gender is shown in Table 1. The prevalence increased significantly with increasing age, and 15.7% of patients 70 years of age or older showed airflow limitation.

Among patients >60 years old, there were 295 non-smokers, 174 former smokers, and 95 current smokers. In addition, airflow limitation was detected

in a significantly higher percentage of men (13.9%) than women (5.3%). The prevalence of airflow limitation according to smoking status and pack-years of smoking is also shown in Table 1. The prevalence of airflow limitation was significantly lower in non-smokers than in former smokers or current smokers, and increased significantly with increasing number of pack-years smoked.

There were no differences in the prevalence of airflow limitation between patients with or without underlying diseases such as hyperlipidaemia, hypertension, or diabetes. However, airflow limitation was detected in only 2.3% (2/89) of the 89 patients who used statins, which was approximately five times lower than the incidence among patients who did not use statins (10.5% (64/609)) (Table 1). There were no

Table 1 Prevalence of airflow limitation according to age, gender, smoking status, pack-years of smoking, and use of statins, angiotensin receptor blockers and angiotensin-converting enzyme inhibitors

			Airflow limitation		
Factor	Category	Number of subjects	Number of subjects	%	*P* value
Age, years	40–49	114	1	0.88	<0.001
	50–59	175	4	2.29	
	60–69	271	30	11.07	
	70–	293	46	15.70	
Gender	Males	416	58	13.94	<0.001
	Females	437	23	5.26	
Smoking status	Current smokers	174	25	14.37	<0.001
	Former smokers	237	31	13.08	
	Never smokers	442	25	5.66	
Pack-years	0–15	528	28	5.30	<0.001
	15–30	113	9	7.96	
	30–45	103	19	18.45	
	45–	109	25	22.94	
Statin use	Yes	89	2	2.25	0.01
	No	609	64	10.51	
ARB use	Yes	93	10	10.75	0.553
	No	522	45	8.62	
ACEI use	Yes	12	1	8.33	1.000
	No	603	54	8.96	

ACEI, angiotensin-converting enzyme inhibitor; ARB, angiotensin receptor blocker.

Table 2 Comparison of patient characteristics between those who did or did not use statins

			Statin use		
Factor		Categories	Yes ($n = 89$)	No ($n = 609$)	*P* value
Age, years, mean ± SD			63.7 ± 9.1	63.3 ± 12.0	0.946
Gender, *n* (%)		Males	30 (33.7)	320 (52.5)	<0.001
		Females	59 (66.3)	289 (47.5)	
Smoking status, *n* (%)		Current smokers	6 (6.7)	136 (22.3)	<0.001
		Former smokers	24 (27.0)	181 (29.7)	
		Never smokers	59 (66.3)	292 (47.9)	
Pack-years, mean ± SD			23.9 ± 16.2	34.8 ± 27.2	0.032
Respiratory symptoms, *n* (%)	Cough	Yes	19 (21.3)	111 (18.2)	0.468
		No	70 (78.7)	498 (81.8)	
	Sputum	Yes	20 (22.5)	132 (21.7)	0.891
		No	69 (77.5)	477 (78.3)	
	Dyspnoea	Yes	16 (18.0)	145 (23.8)	0.281
		No	73 (82.0)	464 (76.2)	
Drug treatment, *n* (%)	ARB	Yes	26 (31.7)	63 (13.3)	<0.001
		No	56 (68.3)	412 (86.7)	
	ACEI	Yes	2 (2.4)	10 (2.1)	0.693
		No	80 (97.6)	465 (97.9)	

ACEI, angiotensin-converting enzyme inhibitor; ARB, angiotensin receptor blocker.

Table 3 Association of statin use with airflow limitation according to smoking status

Smoking status	Statin use	Airflow limitation			Odds ratio
		Present	Absent	Total	
Current smokers, *n* (%)	Yes	0 (0.0)	6 (100.0)	6 (100.0)	Point estimate 0.41†
	No	21 (15.4)	115 (84.6)	136 (100.0)	95% CI: 0.02–7.61†
Former smokers, *n* (%)	Yes	0 (0.0)	24 (100.0)	24 (100.0)	Point estimate 0.12†
	No	26 (14.4)	155 (85.6)	181 (100.0)	95% CI: 0.01–2.03†
Never smokers, *n* (%)	Yes	2 (3.4)	57 (96.6)	59 (100.0)	Point estimate 0.57
	No	17 (5.8)	275 (94.2)	292 (100.0)	95% CI: 0.13–2.53
Test for homogeneity of odds ratio (Breslow–Day test)					$P = 0.267$

† Because cell counts included zero, adjustments were made by adding 0.5 to all cells.

Table 4 Multivariate analysis of factors associated with airflow limitation

Factor	Categories		Odds ratio (95% CI)	*P* value
Age, years	40–49		Reference	
	50–59	1.23	3.43 (0.32–36.97)	0.309
	60–69	2.70	14.95 (1.62–138.0)	0.017
	70–	3.08	21.73 (2.39–197.7)	0.006
Gender	Males	0.33	1.39 (0.46–4.18)	0.563
	Females		Reference	
Smoking status	Current/former smoker	−0.87	0.42 (0.10–1.75)	0.232
	Never smoker		Reference	
Pack-years	0–24		Reference	
	25–49	1.24	3.45 (0.87–13.70)	0.078
	50–	2.02	7.54 (1.77–32.05)	0.006
Number of respiratory symptoms	None		Reference	
	One	0.76	2.15 (0.97–4.74)	0.059
	Two	2.03	7.61 (2.68–21.61)	<0.001
	Three	2.37	10.66 (2.07–54.80)	0.005
	Four	3.08	21.67 (2.41–194.9)	0.006
Statin use	Yes	−1.27	0.28 (0.06–1.28)	0.101
	No		Reference	
ARB use	Yes	0.58	1.78 (0.75–4.24)	0.192
	No		Reference	
ACEI use	Yes	−0.17	0.85 (0.09–8.17)	0.885
	No		Reference	

ACEI, angiotensin-converting enzyme inhibitor; ARB, angiotensin receptor blocker; CI, confidence interval.

significant differences in the prevalence of airflow limitation between patients who did or did not use angiotensin receptor blockers or angiotensin-converting enzyme inhibitors. Table 2 shows a comparison of the demographic characteristics of the patients according to use or non-use of statins. There were no differences in age, respiratory symptoms or the number of patients using angiotensin-converting enzyme inhibitors, between those who used and those who did not use statins. However, the proportions of males, smokers and the pack-years of smoking were significantly lower in the group of patients who used statins.

The prevalence of airflow limitation in patients who used or did not use statins, according to smoking status, is shown in Table 3. Among the 347 patients with a history of past or current smoking, airflow limitation was not detected in the 30 patients who used statins, but was detected in 47 (14.8%) of the 317 patients who did not use statins. On the other hand, among the 351 non-smokers, the prevalence of airflow limitation was 3.4% in those who used statins and 5.8% in those who did not. Multivariate analysis of the factors associated with airflow limitation showed that age, pack-years of smoking and the number of current respiratory symptoms were important factors. However, use or non-use of statins was not associated with airflow limitation (Table 4).

DISCUSSION

In order to determine the prevalence of airflow limitation among patients who visited medical facilities

that provide primary care, a cross-sectional study based on a questionnaire and spirometry was performed. Airflow limitation was detected in 81 (9.5%) of 853 patients, 40 years of age and older. In a previous epidemiological study of the prevalence of COPD in Japan (Nippon COPD Epidemiology Study), the prevalence of COPD was 8.5% among those over the age of 40 years.[21] The prevalence of COPD was slightly higher in the present study but the results were similar. Patients were not asked to inhale bronchodilators before the pulmonary function tests. Therefore, the possibility that patients with chronic airway obstruction, such as undiagnosed asthma, were included among those presenting with airflow limitation cannot be ruled out. However, since the prevalence of airflow limitation was about the same as that in the Nippon COPD Epidemiology Study, it is reasonable to assume that most of the patients with airflow limitation had COPD.

It was reported that the prevalence of subclinical (undetected) COPD among patients receiving routine clinical check-ups differed according to the demographic characteristics of the patient population, such as male : female ratio and percentage of smokers. Koga *et al.*[22] reported that the prevalence of previously unidentified COPD was 7.4% among patients who received a medical check-up, 16.3% among those who visited a primary care facility and 25.8% among those who underwent pre-operative pulmonary function tests. In similar studies of patients visiting primary care facilities,[23,24] the reported prevalence of airflow limitation was 27 and 30%, respectively. In the present study, the prevalence of airflow limitation was lower than that reported in similar previous studies. The low prevalence of COPD in the present study may be explained by the male/female ratio of 416/437 (i.e. almost 1:1), and the relatively low percentage of patients >70 years old (34.3%, 293 patients).

In this study, we also investigated whether the use or non-use of statins influenced the prevalence of airflow limitation. The results showed that the prevalence of airflow limitation among patients who used statins was approximately five times lower than that among patients who did not use statins. Statins play an important role in the prevention and treatment of hyperlipidaemia and atherosclerotic disease. Recent reports indicate that statins have other effects that are independent of their effect in reducing levels of low density lipoprotein cholesterol, such as anti-inflammatory and anti-fibrotic effects, and improvement in endothelial function.[10–12]

Recently, there have been some important reports on the effects of statins in patients with COPD.[13–20] Young *et al.*[6] suggested that the anti-inflammatory effects of statins on both pulmonary and systemic inflammation, through inhibition of guanosine triphosphatase and nuclear factor-κB-mediated activation of inflammatory and matrix remodelling pathways, may result in substantial benefits for patients with COPD. With respect to the effect of statins on airflow limitation, Keddissi *et al.*[25] reported that the annual decline in FEV_1 in 215 former and current smoking patients who used statins (85 ± 171 mL) was significantly less than that in 203 patients who did not use statins (−5 ± 201 mL). Alexeeff *et al.*[26] also reported a follow-up study of elderly men (veterans), including some COPD patients, which showed that statins reduced the rate of decline in FEV_1 with age, although there was a difference in the effect with respect to smoking status. Those results support the results from the present study and suggested that the prevalence of airflow limitation was lower in patients who used statins because statin use reduced the annual rate of decline in FEV_1.

The limitations of this study include possible bias in the selection of patients. The first bias is the healthy user effect. In this study, the subjects were patients over 40 years of age, who had visited a primary care facility due to lifestyle-related diseases. Therefore, the data analysed was for a group of people whose health care was managed in medical facilities, and so might not be generalizable to the general population. Second, there were differences in patient demographics with regard to statin use, and the sample size was small. In addition, this was a cross-sectional study, and there is the possibility of reverse causality between statin use and airflow limitation. That is, patients with airflow limitation might have previously experienced limitation in activities of daily living. Therefore, they might have had less opportunity to experience symptoms related to cardiovascular events, such as chest pain on exertion, with fewer patients therefore using oral statins. Therefore, from the results of this study, it is difficult to determine whether or not statin use directly affects the prevalence of airflow limitation. However, all outpatients attending the selected medical facilities were invited to participate and all patients who consented to participate underwent spirometry. Therefore, we believe that there was no intentional bias in patient selection or exclusion of data.

In conclusion, this cross-sectional study is the first from Japan to demonstrate the potential impact of statin use on the prevalence of airflow limitation. It would appear to be important to accumulate more information on this topic in the future.

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INTERNAL MEDICINE

□ CASE REPORT □

A Rare Case of Asymptomatic Diffuse Pulmonary Ossification Detected during a Routine Health Examination

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Abstract

A 26-year-old man presented at our hospital in 2008 to undergo detailed investigations as part of a routine health examination. Chest computed tomography (CT) showed linear and reticular opacities with, in part, diffuse calcification in the lung fields bilaterally. A surgical lung biopsy was performed and the histological findings were compatible with a diagnosis of diffuse pulmonary ossification (DPO) of the dendriform type. DPO usually occurs as a secondary disease. As the histological changes in interstitial fibrosis were minimal rather than diffuse and not significant enough to be regarded as interstitial pneumonia, we considered this to be an idiopathic case. However, the findings appear to suggest that inflammation and fibrosis were associated with ossification.

Key words: pulmonary ossification, ectopic bone formation

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Introduction

Diffuse pulmonary ossification (DPO) is a rare disease characterized by ectopic bone formation within pulmonary tissue. Living cases are rarely encountered; most are diagnosed at autopsy. DPO is usually associated with cardiovascular or respiratory disease. Therefore, the number of idiopathic cases is very small (1-4). We herein present an idiopathic case of DPO detected during a routine health examination.

Case Report

A 26-year-old man presented at our hospital in 2008 to undergo detailed investigations as part of a routine annual health examination. An abnormality on chest radiograph had been noted during the previous annual check-up in 2007. The patient was almost asymptomatic. His medical history included a cleft lip and palate (surgically repaired at 6 years of age) and chronic sinusitis (surgically treated at 10 years of age). He did not smoke until 20 years of age; his Brinkman index was 80. His grandmother had suffered from pulmonary tuberculosis; however, there was no other significant family medical history, including genetic diseases or consanguineous marriage. The patient worked in an office-based job as a forwarding agent and had no past or present exposure to any relevant environmental factors.

A physical examination revealed no respiratory or cardiovascular abnormalities. The patient's blood pressure was 126/80 mmHg, his heart rate was 80 bpm, his respiratory rate was 20/min and his percutaneous oxygen saturation was 98% (on room air). No murmurs were detected and normal vesicular sounds (without rales) were heard on auscultation.

Peripheral blood and urinary tests showed slight elevations of urinary deoxypyridinoline (8.7 nmol/mmol · creatinine, normal range is below 7.7) and serum cross-linked carboxyterminal telopeptide of type I collagen (I-CTP) (4.6 ng/mL). The results of pulmonary function tests were normal and electrocardiogram and echocardiogram showed no

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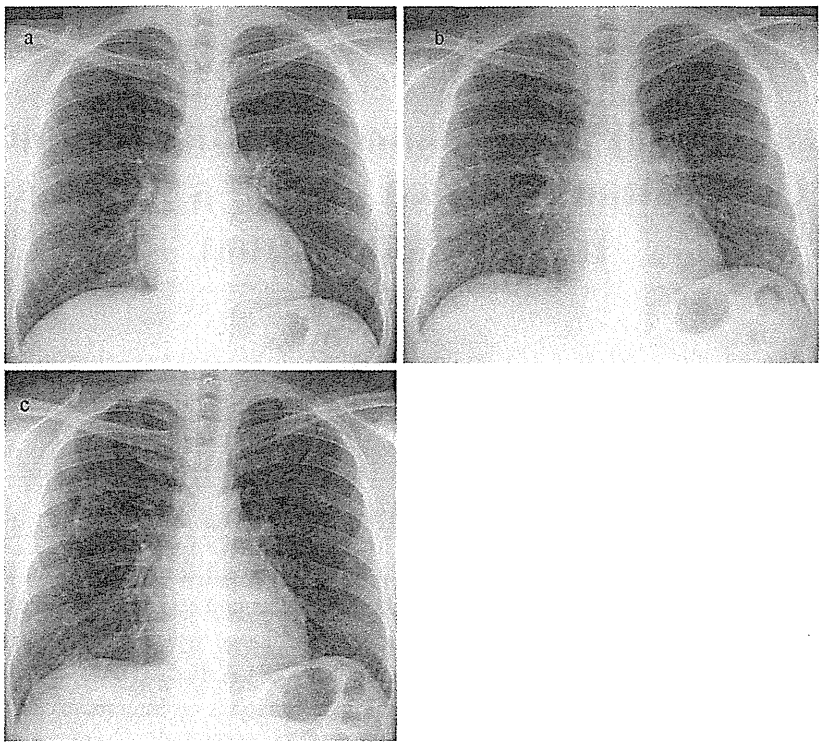

Figure 1. Chest radiographs taken in 2005 (a), 2007 (b) and on admission in 2008 (c) (these chest radiographs were taken under different conditions). A chest radiograph taken on admission showed small, diffuse linear opacities in the lung fields bilaterally. The images showed almost normal findings in 2005. The abnormalities first appeared in 2007 and had become apparent in 2008.

abnormalities.

Chest radiography performed on admission showed small, diffuse linear opacities in the lung fields bilaterally (Fig. 1c). Chest X-ray performed in 2005 showed almost normal findings (Fig. 1a); however, abnormalities appeared in 2007 (Fig. 1b) and became exacerbated by 2008 (Fig. 1c). Computed tomography (CT) demonstrated linear and reticular opacities with diffuse calcification in all lung fields bilaterally (Fig. 2a, b). The distribution was partly posterior dominant. The CT opacities were similar to bone (Fig. 2c). Bone scanning with Tc-99m hydroxymethylene disphosphate showed increased tracer uptake in the lung fields bilaterally (Fig. 3).

Bronchoalveolar lavage fluid (BALF) testing and a transbronchial lung biopsy (TBLB) showed no significant findings. Therefore, we performed a video-assisted thoracoscopic surgery (VATS) lung biopsy.

The histological findings obtained from the VATS-biopsy of rt.S^2 (Fig. 4) showed dendriform mature bone formations with marrow in the alveolar spaces. Although there was some evidence of interstitial fibrosis, the fibrosis was minimal rather than diffuse and not significant enough to be regarded as interstitial pneumonia. Consequently, we diagnosed the patient with idiopathic DPO of the dendriform type.

Discussion

We herein report an asymptomatic case of idiopathic DPO diagnosed on VATS-biopsy that was detected during a routine, annual health examination. DPO is characterized by ectopic bone formation. Living cases are rarely encountered; most are diagnosed at autopsy (1-4). DPO was first reported by Luschka in 1856 (5), and Tseung and Duflou defined DPO as the presence of disseminated, widespread or extensive bone formation in the lungs (1). The etiology of this disease remains uncertain. However, DPO is thought to be associated with inflammation and resulting anoxia, which produces an acidemic environment leading to free radical initiation and propagation (4).

We performed a VATS biopsy on our patient, and a histological pattern of dendriform mature bone formation with marrow was seen in the alveolar spaces. DPO is categorized into two different types: namely, dendriform and nodular. Dendriform ossification is characterized by branching bony spicules in the alveoler septa that usually contain fat marrow (2, 4). Dendriform ossification is seen in patients with chronic pulmonary disease, idiopathic pulmonary fibrosis (6), acute respiratory distress syndrome, chronic obstructive pulmonary disease, organizing pneumonia, rare earth pneumoconiosis or asbestosis or heavy metal exposure (1). Although we could not completely exclude the possibility of secondary DPO following interstitial pneumonia, the histological changes in interstitial fibrosis were minimal rather than diffuse and not significant enough to be regarded as interstitial pneumonia. Therefore, we diagnosed the patient with idiopathic DPO of the dendriform type.

This case involves a very young patient, a 26-year-old

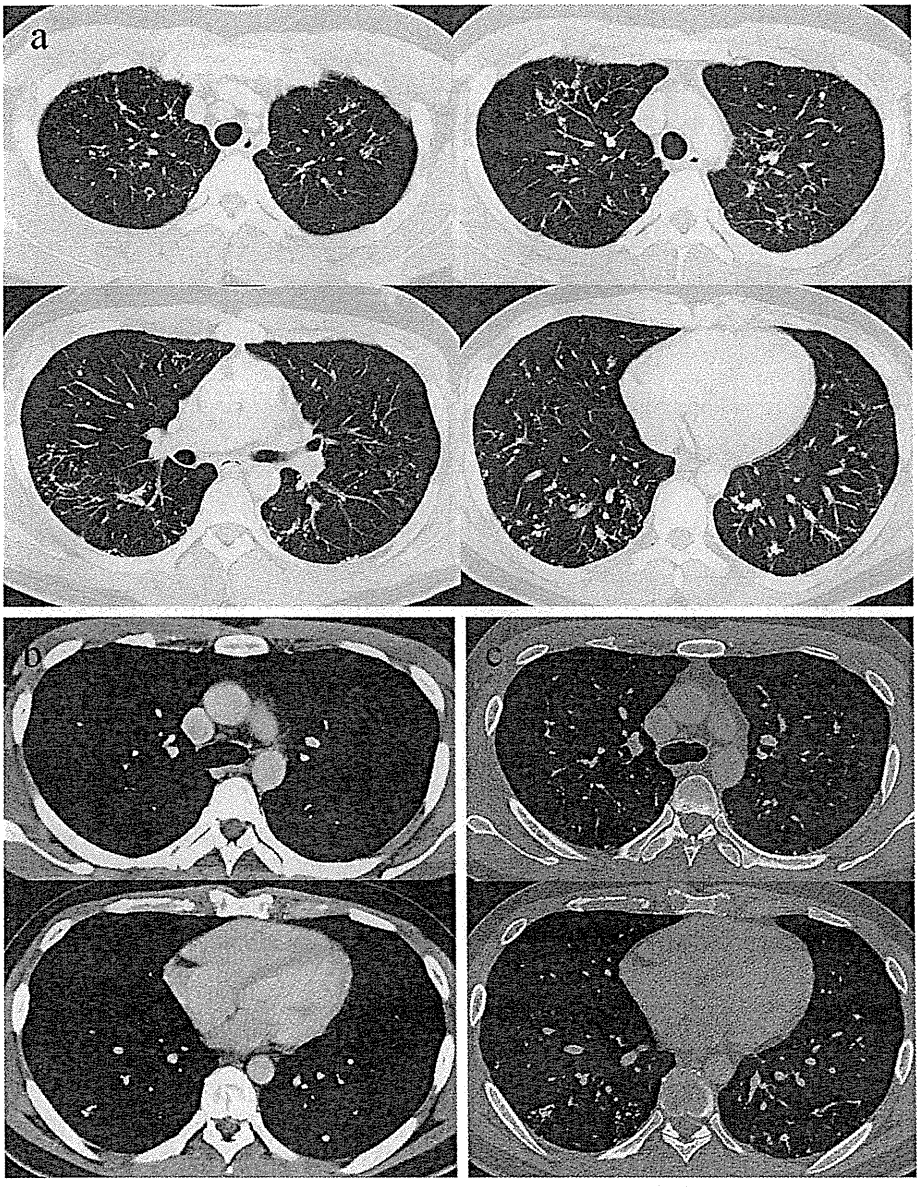

Figure 2. Computed tomography (CT) performed on admission. CT images demonstrated linear and reticular opacities with, in part, diffuse calcification in all the lung fields bilaterally (a, b). The CT opacities were similar to bone (c).

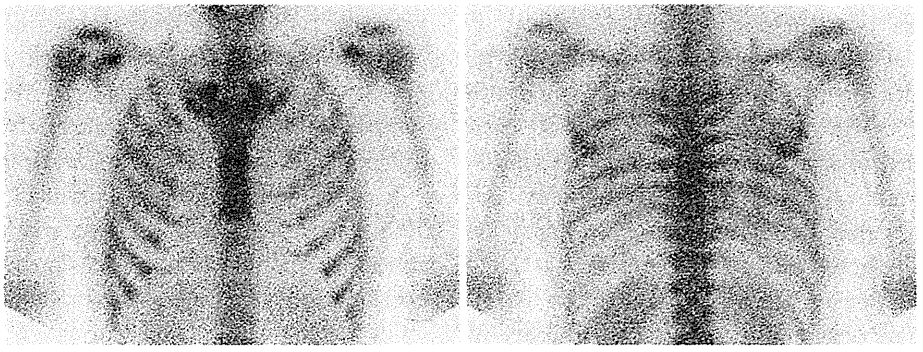

Figure 3. Bone scanning with Tc-99m hydroxymethylene disphosphate. An increased tracer uptake is visible in the lung fields bilaterally.

man. Most reported cases of dendriform ossification have occurred in older men with an average of 67 years of age (7). Azuma et al. reported the phenomenon of familial clustering of dendriform ossification, which suggests a genetic predisposition for the pathogenesis of this disease (7).

The clinical course of dendriform ossification is consid-

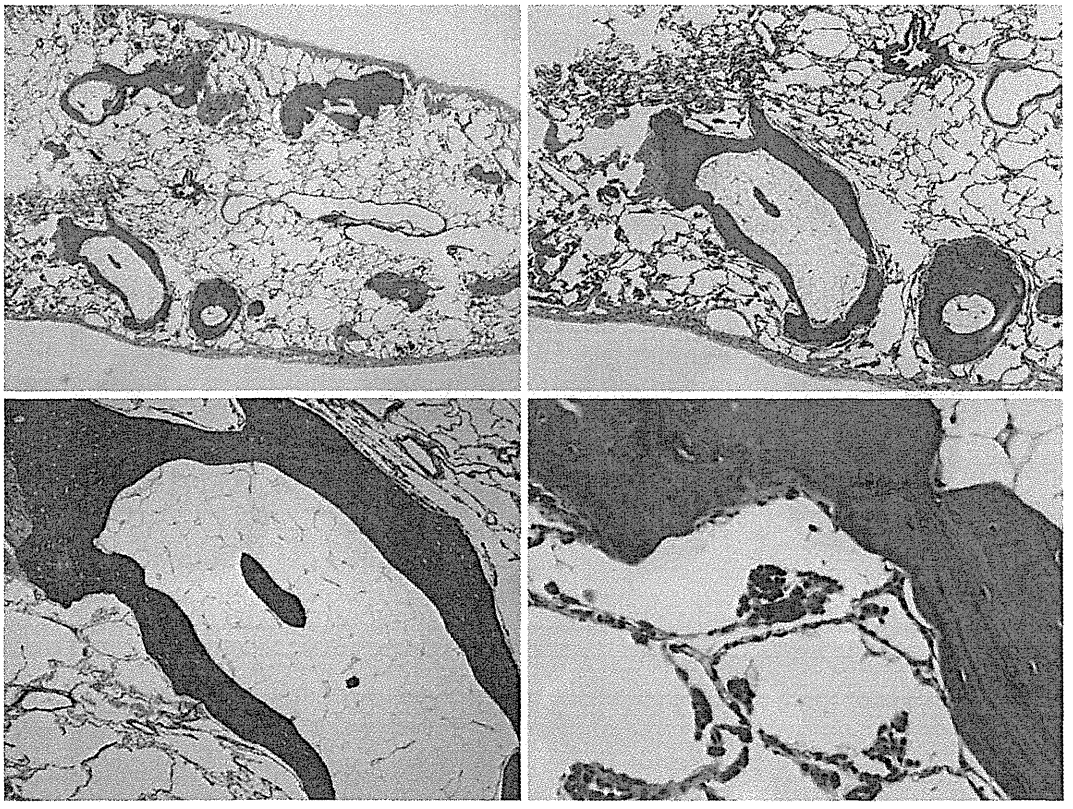

Figure 4. Histological findings from a video-assisted thoracoscopic surgery (VATS) biopsy of rt.S^2 (Hematoxylin and Eosin staining). Dendriform mature bone formations with marrow were seen in the aleveolar spaces. Only minimal interstitial fibrosis was observed in the alveolar septum and haemosiderin-laden macrophages were present in the alveolar spaces. In addition, the fibrosis was not diffuse.

ered to be indolent or slowly progressive (2). Fortunately, the patient's annual check-ups provided us with a rare opportunity to track the progression of the DPO on chest radiographs from the time when the images had shown almost normal findings. Linear and reticular opacities were seen on high resolution computed tomography (HRCT), some of which appeared to have branching forms. This case also showed increased Tc-99m hydroxymethylene tracer uptake in the lung fields bilaterally. In dendriform ossification, HRCT shows a coral-like dendritic pattern (2). Saks et al. reported a case of DPO detected using bone scanning with Tc-99m hydroxymethylene in 1984 (8). However, it has also been reported that Tc-99m hydroxymethylene is less sensitive in the nonextensive stage (2). In this case, the laboratory findings showed slight elevations of urinary deoxypyridinoline and serum I-CTP, which usually imply the predominance of bone resorption. Previous reports of DPO show no diagnostic value in laboratory findings (2). Therefore, at present, we are unable to explain the significance of the laboratory findings in this case.

Although this case was diagnosed clinically as idiopathic, there might be indirect evidence and scientific scenarios to explain inflammation mediated heterotopic ossification. In heterotopic ossification, osteoblasts demonstrate the coexpression of endothelial and osteogenic markers (9). Endothelial cells transdifferentiate into the mesenchymal stem cells (MSCs) that subsequently form ectopic bone in a process called endothelial-to-mesenchymal transition (EndoMT) (9). Transforming growth factor (TGF) β-1 and β-2 induce EndoMT. Myofibroblasts in fibrotic tissues are derived from the expansion and activation of resident tissue fibroblasts, epithelial-mesencymal transition (EMT) and tissue migration of bone marrow-derived circulating fibrocytes (10). Recently, EndoMT has emerged as another possible source of tissue myofibroblasts. Both EMT and EndoMT can be induced by TGF-β (10). EndoMT has been reported to be an important mechanism underlying the process of pulmonary fibrosis (10). EndoMT becomes a common pathway between heterotopic ossification and pulmonary fibrosis. This might explain the evidence of minimal interstitial fibrosis.

In conclusion, we herein presented a case of idiopathic DPO that was detected during a routine annual health examination. The patient's progression was followed over the preceding four years using chest radiographs.

The authors state that they have no Conflict of Interest (COI).

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