macrophages, with less accumulation of proteoglycan than did wild constructs. Analysis of the co-culture medium identified G-CSF as an inducer of FasL on chondrocytes, and G-CSF-treated tissue-engineered cartilage showed less infiltration of macrophages, with increased formation of cartilage after transplantation. The interactions between chondrocytes and macrophages may increase G-CSF secretion in macrophages and induce FasL on chondrocytes, which in turn induce the apoptosis of macrophages and suppress tissue reactions, promoting the maturation of tissue-engineered cartilage. These findings provide scientific insight into the mechanism of autologous chondrocyte transplantation, which could be applied as a novel strategy for cartilage tissue engineering.

INTRODUCTION

Cartilage regenerative medicine using autologous chondrocyte transplantation (ACT) has been available since the 1980s, and more than 15,000 patients have received this treatment for focal cartilage defects in joints. Recently, some groups have expanded the indication for ACT to nasal augmentation, in which chondrocytes in a gelatinous chondroid matrix were injected into subcutaneous pockets in the nose [1]. We have also started a clinical study of ACT-based treatment using a biodegradable polymer for severe nasal deformity in patients with cleft lip and palate (JPRN-UMIN000005472). Since most of these cartilaginous diseases and deformities are irreversible and cannot be fully restored by conventional transplantation of autologous tissues or artificial materials, ACT-based treatment should be more prevalent. To broaden the application of ACT to severe defects in joint or maxillofacial cartilage, combined usage with a scaffold would be necessary to provide a proper morphologic shape and rigidity. Such scaffolds can be fabricated by employing biomaterials, such as biodegradable polymers.

ACT-based treatment uses autologous cells, and thus is advantageous to minimize the risk of immunogenic rejection. In studies using mice or beagles, however, when tissue-engineered constructs consisting of a biodegradable scaffold and syngenic or autologous chondrocytes were transplanted into subcutaneous pockets, some of them developed severe, prolonged tissue reactions [2, 3]. Histologically, accumulation of macrophages and abundant fibrosis were found in and around these transplants, with the formation of foreign body granulomas. Therefore, one of the requirements for successful transplantation of tissue-engineered cartilage is to control inflammation.

Meanwhile, in the case of successfully regenerated cartilage by transplanted chondrocytes in mice or beagles, chondrocytes were shown to produce local factors that could inhibit the actions of macrophages. In our previous study [2, 3], tissue-engineered cartilage constructs consisting of mouse auricular chondrocytes and a biodegradable scaffold were transplanted into EGFP-transgenic mice with the same genetic background. The host-derived cells, distinguished by the positivity of EGFP

fluorescence, were mostly macrophages, and decreased and were confined to non-cartilage areas after an initial increase. The chondrocytes expressed some factors related to immune privilege including Fas ligand (FasL) and transforming growth factor-β (TGF-β), suggesting that chondrocytes in tissue-engineered cartilage may suppress the actions of macrophages by inducing immune privilege with these factors.

Immune privilege exists physiologically in certain tissues such as the eye, brain, ovary, testis and pregnant uterus, where antigen normally does not initiate an immune reaction. The molecular mechanisms maintaining immune privilege in these tissues are considered to be a lack of lymphatic drainage, the presence of a physical barrier and the production of immunosuppressive cytokines/neuropeptides [4]. Apart from the physiological immune privilege mentioned above, recent research has revealed another type of immune privilege, which is induced in pathological conditions. Hepatocytes do not express FasL, but FasL could be induced in hepatocellular carcinomas, promoting infiltration and growth in surrounding tissues [5]. Also, FasL becomes more detectable in hepatocytes in alcoholic hepatitis, liver allograft rejection, and Wilson's disease [6], suggesting that hepatocytes may have increased expression of FasL in pathological conditions. In another study, immunization was shown to induce the expression of functional FasL in the liver and small intestine [7], indicating that strong immune responses trigger the expression of FasL in some tissues. Such inducible peripheral, non-lymphoid FasL is considered to mitigate the tissue damage caused by strong immune response [4]. Considering that factors related to immune privilege were increased in tissue-engineered cartilage after transplantation in mice and beagles [2, 3], immune privilege may be induced in tissue-engineered cartilage as well by the surrounding immunological environment after transplantation.

In the present study, we investigated the involvement of immune privilege in the transplantation of tissue-engineered cartilage, using mouse auricular chondrocytes. Among the mediators involved in immune privilege, we especially focused on FasL, and investigated its function in tissue-engineered cartilage using FasL-dysfunctional mice, C57BL/6JSlc-gld/gld (gld). The gld (generalized lymphoproliferative disease) mice are known to have a point mutation within the *Fasl* gene, resulting in the expression of non-functional FasL [8]. Furthermore, molecules that increased the expression of FasL on the auricular chondrocytes were identified, which were then applied in the subcutaneous transplantation of tissue-engineered cartilage in mice, to verify their effects in regulating tissue reactions and subsequent maturation of tissue-engineered cartilage.

MATERIALS AND METHODS

Materials

Dulbecco's Modified Eagle's Medium: Nutrient Mixture F-12 (DMEM/F12), penicillin-streptomycin solution, and trypsin-EDTA solution were purchased from

Sigma Chemical Co. (MO, USA). Collagenase from Clostridium histolyticum was from Wako Pure Chemical Industries (Osaka, Japan), insulin was from MP Biomedicals (Irvine, CA, USA), and FGF-2 was from Kaken Pharmaceutical Co., Ltd. (Tokyo, Japan). Other materials included 3% atelocollagen implants from Koken Co., Ltd. (Tokyo, Japan), poly-L-lactic acid (PLLA) porous scaffolds from KRI (Kyoto, Japan), anti-type II collagen antibody from LSL (Tokyo, Japan), anti-F4/80 antibody (Cl: A3-1) from BMA (Augst, Switzerland), anti-fas ligand (FasL) antibody from GeneTex Inc. (TX, USA), anti-STAT3 antibody and anti-phospho STAT3 from Cell Signaling Technology Japan, K.K. (Tokyo, Japan). Recombinant mouse G-CSF, IL-6, IP-10, KC, MIP-1α, MIP-1β and MIP-2 were from R&D Systems, Inc. (MN, USA). The biotinylated secondary antibody, Vectastain Elite ABC Kit and Peroxidase Substrate kit DAB were all obtained from Vector Laboratories (CA, USA). CytoTox 96® Non-Radioactive from Promega K.K. (Tokyo, Japan) and FITC Annexin V Apoptosis Detection Kit II from BD (NJ, USA) were used. C57BL/6J (wild) and C57BL/6JSlc-gld/gld (gld) were purchased from Japan SLC, Inc. (Shizuoka, Japan).

Cell culture

Human cartilage was obtained under informed consent from remnant auricular cartilage of microtia patients who underwent surgery at Nagata Microtia and Reconstructive Plastic Surgery Clinic. All the procedures for the present experiments were approved by the ethics committee (ethics permission #622, 752, 753) or institutional committee for animal research of the University of Tokyo Hospital, and the experiments was conducted according to the principles expressed in the Declaration of Helsinki. Isolation of human auricular cartilage and culture of chondrocytes were conducted as previously described [9]. Mouse auricular chondrocytes were obtained as described previously [2]. Briefly, the ear and external ear canal of wild and gld mice were digested with 0.15% collagenase solution for 8 hr, and the isolated chondrocytes were cultured in DMEM/F12 supplemented with 5% FBS, 5 μg/ml insulin and 100 ng/ml FGF-2. Both human and mouse auricular chondrocytes were cultured to passage 2. Mouse macrophage-like cell line RAW264 was purchased from the Riken Cell Bank (Tsukuba, Japan) and maintained in MEM supplemented with 10% FBS and NEAA.

Fabrication of tissue-engineered cartilage and transplantation

PLLA scaffolds, which were produced by sugar-leaching method, were used. The molecular weight of the scaffolds was 200,000, average pore size was 0.3 mm, and average porosity was more than 95%. PLLA scaffolds of 4 x 4 x 3 mm^3 were sterilized in 70% ethanol before use. Then, 1% atelocollagen gel, which was diluted from original 3% atelocollagen gel with DMEM/F12, was used as a cell suspension buffer to retain the chondrocytes in the scaffolds efficiently. To make tissue-engineered constructs, mouse chondrocytes suspended in 1% atelocollagen gel (2 x 10^7 cells/200 μl) were applied to the PLLA scaffolds and incubated at 37°C in 5% CO_2 for 2 hr. Regarding the transplantation procedure, wild mice were anesthetized by

intraperitoneal injection of sodium pentobarbital (50 mg/kg). A small incision was made on the back in the midline, and two types of constructs, which contained either wild-derived or gld-derived chondrocytes, were transplanted subcutaneously in each animal. Two or 8 weeks after the operation, the constructs were harvested and cut into equal parts (n=8); one piece was frozen in liquid nitrogen and preserved at -80°C for biochemical analysis, and the other piece was fixed in 4% paraformaldehyde for 3 hr, embedded in paraffin, and cut into 8-μm sections for histological and immunohistochemical analysis. In some experiments, the constructs were treated with G-CSF (35 pg/mL) in re-differentiation medium[10] for 5 days, and then transplanted subcutaneously as described above.

Biochemical analysis

Samples stored at -80°C for biochemical analysis were cut into small pieces with scissors, and suspended in10 mg/mL pepsin, 0.05 M acetic acid. After digestion at 4°C for 48 hr, 1 mg/mL pancreatic elastase, 0.1 mM Tris, 0.02 M NaCl, 5 mM $CaCl_2$ (pH 7.8 - 8.0) was added, and the samples were kept at 4°C overnight. The samples were then centrifuged at 9100 x g for 5 min, and the sulfated GAG content in the supernatant was measured using Alcian blue binding assay (Wieslab AB, Lund, Sweden) according to the manufacturer's instructions. Measurement was performed three times, and the mean values were used for statistical analysis.

Histological and immunohistochemical staining

The sections were stained with toluidine blue to detect proteoglycan, as well as with hematoxylin and eosin (H-E staining). The sections were also used for immunohistochemical staining for F4/80, FasL, Fas and CD3ε[9].

Cytotoxic analysis and apoptosis detection

Mouse auricular chondrocytes (wild or gld) and a mouse macrophage cell line, RAW264, were co-cultured at various ratios for 4 hr, and cytotoxicity was measured using CytoTox96 Non-Radioactive Cytotoxicity Assay (Promega, Osaka, Japan). When activated macrophages were required, RAW264 were treated with LPS (1 μg/mL) and IFN-γ (10 ng/mL) for 24 hr[11]. For the detection of apoptosis in macrophages, the co-cultured cells (chondrocytes:RAW264=19:1) were stained with anti-F4/80 antibody, and the positivity of annexin V and PI in F4/80-positive cells was analyzed by flowcytometry using BD™ LSR II.

RNA isolation and real-time RT-PCR

A mixture of mouse auricular chondrocytes and RAW264 (chondrocytes:RAW264=19:1) or chondrocytes alone in 1% atelocollagen gel were respectively embedded in PLLA scaffolds, and cultured in re-differentiation medium [10] for 7 days. Collected media were used for Proteome Profiler Array (R&D Systems Inc., MN), and secreted cytokines were quantified as the relative ratio by measuring the blotted areas. Subsequently, factors that increased in co-culture by 100 relative ratios were chosen as possible inducers of FasL. Human auricular

chondrocytes embedded in 1% atelocollagen gel (2 x 10^5 cells/20 μL) were cultured in re-differentiation medium containing the recombinant protein of the chosen factor. The concentration of each factor added to the medium was ED_{50}, which was provided by the manufacturers, as well as one tenth ED_{50} and 10 times ED_{50}. Five days later, total RNA was isolated with Isogen, and then reverse-transcribed with reverse transcriptase and random hexamers. Gene expression was detected by real-time RT-PCR using the standard SYBR green method with a 7500 fast real-time PCR system (Applied BioSystems, CA, USA). Standard templates were produced according to a previous study[10]. Transcript levels were normalized to that of *GAPDH*.

Western blotting

Human auricular chondrocytes embedded in 1% atelocollagen gel (2 x 10^5 cells/20 μL) were incubated for 2 hr before addition of 2 mL re-differentiation medium[10]. Some media contained G-CSF (35 pg/mL) and a JAK2 kinase inhibitor, AG490 (4 x 10^{-3} mol/L). After 10 and 30 minutes, the cells were harvested with M-PER, and the supernatant was used for western blotting to detect the expression of STAT and p-STAT according to previous studies [10].

Statistics

Data are expressed as mean ± SD. Statistical significance was evaluated using Student's t-test. A value of $p < 0.05$ was interpreted to denote statistical significance.

RESULTS

Localization of macrophages and Fas/FasL during in vivo cartilage regeneration

We made tissue-engineered cartilage constructs using C57BL/6 mouse auricular chondrocytes, and syngenically transplanted them into the backs of host mice [2]. Toluidine blue staining showed an increase in metachromatic areas by 8 weeks, indicating accumulation of proteoglycan and maturation of cartilage (Fig. 1A; TB). Immunohistochemical staining for COL2 also confirmed the maturation of transplanted cartilage (Fig. 1A; COL2). Meanwhile, F4/80-positive macrophages were prominent at 2 weeks, which then decreased and were localized in non-cartilage areas by 8 weeks (Fig. 1A; F4/80). FasL was immunolocalized on chondrocytes in the tissue-engineered transplants, and was especially intense at 2 weeks, the early stage of cartilage maturation (Fig. 1A; FasL). In contrast, cells positive for Fas were mainly macrophages, and were sparsely scattered throughout the observation period (Fig. 1A; Fas). We then made human tissue-engineered cartilage constructs and transplanted them into nude mice to examine the expression of *FASL* and *FAS* by real-time RT PCR using human-specific primers. *FASL* was enhanced in chondrocytes of tissue-engineered cartilage at 2 and 8 weeks after transplantation, while expression of *FAS* was decreased 8 weeks after transplantation (Fig. 1B; *FASL* and *FAS*). These findings suggest that FasL on chondrocytes may induce cell death in Fas-expressing macrophages and regulate tissue reactions.

Chondrocytes reduce viability of macrophages

To examine the function of FasL expressed on chondrocytes, we prepared chondrocytes from C57BL/6J (wild) and FasL hypomorphic mice, C57BL/6J-gld (gld). As macrophages have been identified as the major component of the host reaction [2], we co-cultured chondrocytes with mouse macrophage-like cells, RAW264, at a ratio of 10-1.25:1, and examined the induction of cell death and apoptosis by cytotoxicity analysis and flow cytometry. Cytotoxicity analysis revealed that wild-chondrocytes induced more cell death in RAW264 than did gld-chondrocytes, when the ratio of chondrocytes to RAW264 was as high as 10:1 (Fig. 2A), suggesting that FasL on chondrocytes can induce cell death in co-cultured macrophages. The difference in cytotoxicity of chondrocytes between wild and gld mice, however, became unrecognizable when the ratio of chondrocytes to RAW264 decreased. Since activated macrophages are known to have increased expression of Fas and thus be more apoptotic [12], we activated RAW264 with LPS and IFN-γ to see if this could increase the cell death of RAW264 in the co-culture. As expected, activated RAW264 in the co-culture underwent a higher rate of cell death compared to RAW264 (Fig. 2A). Similarly, in flow cytometric analysis of apoptosis, RAW264 showed increased positivity of annexin V (a marker of early apoptosis) and double positivity of annexin V and PI (a marker of late apoptosis) when co-cultured with wild-type chondrocytes at a chondrocyte:macrophage ratio of 10:1 (Fig. 2B). The viability of both types of chondrocytes was hardly affected by RAW264, regardless of the activation level of RAW264 (Fig. 2C). However, analysis of gene expression by real-time RT-PCR revealed that the expression of *COL 2* in chondrocytes was decreased and that of *COL1* was enhanced as the ratio of RAW264 increased (Fig. 2D). These findings indicate that chondrocytes decreased the viability of macrophages, while macrophages did not affect the viability of chondrocytes. Instead, macrophages reduced the production of cartilage matrix by chondrocytes, possibly by secreting catabolic factors.

FasL on chondrocytes promotes maturation of tissue-engineered cartilage

To examine how the expression of FasL on chondrocytes could affect in vivo regeneration of tissue-engineered cartilage, we made tissue-engineered cartilage constructs using wild or gld chondrocytes, and syngenically transplanted them into the back of wild mice. HE staining and toluidine-blue staining of the tissue-engineered cartilage constructs revealed suppressed maturation of cartilage and less accumulation of extracellular matrix in gld constructs at both 2 and 8 weeks (Fig. 3A; HE and TB). The content of GAG was also significantly decreased in gld-constructs (Fig. 3B). Speculating that FasL dysfunction in gld-chondrocytes could decrease the apoptosis of macrophages in tissue-engineered cartilage, resulting in the increase of surviving macrophages, we conducted immunohistochemical staining for F4/80 antigen to evaluate the localization and number of macrophages (Fig. 4A). Both wild and gld constructs exhibited infiltrating macrophages throughout the constructs at 2 weeks, which

subsequently decreased and persisted only in non-cartilage areas by 8 weeks. However, the tissue-engineered cartilage of gld mice showed more prominent accumulation of macrophages than did wild constructs. Indeed, the macrophage area measured by DAB positivity in immunohistochemical staining for F4/80 showed that macrophage area was significantly increased in gld constructs at 2 weeks. It was therefore considered that FasL on chondrocytes may induce apoptosis of macrophages and suppress tissue reactions, eventually promoting the maturation of tissue-engineered cartilage.

G-CSF induces FasL expression on chondrocytes

We then searched for possible molecules that induce the expression of FasL on chondrocytes. In vitro analysis of a 3D culture of chondrocytes under differentiation stimuli [10] showed that the differentiation of chondrocytes and *FASL* expression were inversely proportional (Supplemental Fig. 1). Next, we speculated that the enhanced expression of FasL in chondrocytes could be attributable to macrophages. Indeed, co-culture of chondrocytes and RAW264 embedded in atelocollagen gel increased the expression of *FASL* on chondrocytes (Fig. 4B). Furthermore, in tissue-engineered cartilage, double immunohistochemical staining for FasL and F4/80 demonstrated slight positivity of FasL in chondrocytes where macrophages were closely, but not contiguously localized (Fig. 4C). These results suggest that macrophages are required to induce FasL expression in chondrocytes, and that macrophages may secrete some factors inducing FasL on chondrocytes.

Presuming that the co-culture medium of chondrocytes and RAW264 could include such molecules, we conducted a proteome array using the culture media of chondrocytes, RAW264 and co-cultured chondrocytes and RAW264. In the proteome array of 40 inflammation-related cytokines, 17 cytokines were detected in the co-culture medium (Fig. 5A), while 7 cytokines were increased by more than 100 relative values compared to the medium of chondrocytes alone (Fig. 5A; Secretion of cytokines, asterisks). Further gene expression analysis using real-time RT-PCR revealed that G-CSF, IL-6, KC, MIP-1α and MIP-1β enhanced the expression of *FasL* in chondrocytes embedded in atelocollagen gel (Fig. 5B). Among them, IL-6 at a concentration of ED_{50} did not affect the expression of *FasL*, in spite of marked enhancement at one-tenth ED_{50}, while G-CSF showed a concentration-dependent proportional effect on the expression of FasL. When these two factors were respectively added to the culture of tissue-engineered cartilage, the expression of FasL was detected on chondrocytes also at the protein level by immunohistochemical staining, although the effect was more marked with G-CSF (Fig. 5C). Also, the receptors for these factors, including G-CSFR, were confirmed to be expressed on cultured human chondrocytes (Fig. 5D). Therefore, we considered that G-CSF signaling could be involved in the up-regulation of FasL. Indeed, human chondrocytes in 3D culture demonstrated an elevated level of phospho-STAT3 at 10 and 30 min after adding G-CSF, while the effect was abolished by the addition of AG490, a tyrosine kinase inhibitor that inhibits JAK-STAT signaling (Fig. 5E). Furthermore, human

chondrocytes in 3D pellets showed increased expression of *FASL* when cultured in medium containing G-CSF, although the effect was also diminished by the addition of AG490 (Fig. 5F). To see the time-course effects of G-CSF on the expression of FasL in chondrocytes, chondrocytes in 1% atelocollagen gel were cultured for 5 days in medium containing G-CSF, and then the medium was changed to medium devoid of G-CSF. In real-time RT-PCR, the expression of *FASL* in chondrocytes was increased by treatment with G-CSF for 5 days, and continued to increase for the next 3 days without G-CSF (Supplemental Fig. 3A). Meanwhile, immunohistochemical staining for FasL demonstrated sustained expression of FasL even after 14 days (Supplemental Fig. 3B), indicating that G-CSF-treated chondrocytes could express FasL without continuous stimulation with G-CSF at least for several days. These results suggest that G-CSF, which was secreted during the interaction between chondrocytes and macrophages, could enhance the expression of FasL on chondrocytes.

Application of G-CSF in transplantation of tissue-engineered cartilage

To explore the possible application of G-CSF in cartilage tissue engineering, recombinant protein of G-CSF was applied to the medium during the incubation of tissue-engineered constructs consisting of mouse chondrocytes for 5 days before transplantation. Compared with non-pretreated control constructs, G-CSF-pretreated constructs exhibited enhanced accumulation of cartilaginous matrix at 2 weeks after transplantation (Fig. 6A; TB, COL2 and Fig. 6B). Immunohistochemical staining for F4/80 revealed less infiltration of macrophages in G-CSF-pretreated constructs (Fig. 6A; F4/80), which also supports the efficacy of G-CSF.

DISCUSSION

Inflammatory reactions against tissue-engineered cartilage using autologous chondrocytes were mediated mainly by macrophages. In this study, tissue-engineered cartilage constructs containing FasL-dysfunctional chondrocytes (gld) showed more intense infiltration of macrophages than those containing wild-type chondrocytes, suggesting that FasL on chondrocytes could create an immunologically privileged environment against macrophages. Classical immune privilege that exists physiologically, such as in anterior chamber of eye and brain, is considered to protect tissues, where overly activated T cells could deteriorate anatomical structure of the tissues, directly leading to the loss of functions. Immune privilege in tissue-engineered cartilage, however, could be induced by the immunological stimulation after transplantation, and it mainly serves to inhibit the localization of macrophages, promoting the maturation of tissue-engineered cartilage.

However, inducible immune privilege may not be so critical for the survival of tissue-engineered cartilage, since gld constructs still formed cartilage even without FasL-associated immune privilege (Fig. 3). Nonetheless, wild-type constructs showed increased accumulation of cartilaginous matrix, so immune privilege induced in tissue-engineered cartilage is advantageous to

promote cartilage maturation by suppressing the localization of macrophages. Macrophages produce various enzymes, complement factors, and other inflammatory cytokines, which potentially decrease the accumulation of cartilage matrix, hampering the regeneration of engineered tissues. Indeed, in the present study, chondrocytes co-cultured with RAW264 showed decreased expression of *COL2*, though their viability was not so affected. It is reported that the catabolic cytokine, IL-1β, had the potential to induce chondrocytes to secrete aggrecanase and MMPs, causing a loss of proteoglycan in cartilage [13, 14]. This action of IL-1β suggests that even if macrophages do not affect the viability of chondrocytes, their localization could still be detrimental to matrix production in chondrocytes, affecting the maturation of tissue-engineered cartilage.

While the viability of chondrocytes was not so affected by co-cultured macrophages, the viability of macrophages was decreased by chondrocytes. To understand the molecular mechanisms of this event, we may need to consider the subsets of macrophages. Recent studies have classified macrophages mainly into two subsets; classically activated macrophages (M1-type) that basically stimulate immune response, and alternatively activated macrophages (M2-type) that are anti-inflammatory and involved in tissue repair [15, 16]. Considering these phenotypic differences, macrophages that initially infiltrated into tissue-engineered cartilage were speculated to be predominantly M1-type. Therefore, for effective cartilage regeneration, it would be desirable to suppress macrophages when M1-type is dominant. Conveniently, our data (Fig. 2 A) indicated that activated macrophages (M1) were more susceptible to cell death than were inactivated macrophages (M2) when co-cultured with wild-type chondrocytes. This was presumably due to enhanced expression of Fas in M1 macrophages [12], which may make them more easily affected by FasL on chondrocytes. In addition, the viability of macrophages was decreased more markedly by wild-type chondrocytes than by gld-type chondrocytes, suggesting that macrophages became apoptotic by FasL on chondrocytes. Therefore, it was suggested that the expression of FasL on chondrocytes was involved with decreasing the localization of macrophages, resulting in the promotion of cartilage maturation.

Regarding the mechanisms of FasL upregulation in chondrocytes of tissue-engineered constructs, transplantation into the body seemed to be a trigger, because cultured chondrocytes seldom expressed FasL before transplantation. Meanwhile, FasL is constitutively expressed in physiologically immune-privileged sites, such as in cells of the anterior chamber of the eye, neurons and astrocytes of the central nervous system [17]. Regarding the signals for inducing FasL, previous studies have reported that T-cell-receptor (TCR)/CD3 [18, 19], CD28 [20, 21], CD40, stress signaling [22, 23] and IFN-γ[24-26]could initiate the expression of FasL in T cells. In pathological hepatocytes, the expression of FasL was upregulated by virus or CD40. Unlike these previous observations, the present study identified G-CSF and IL-6 as inducers of FasL in chondrocytes, and both of them stimulate

JAK/STST signaling. Generally, G-CSF is known to act as a regulator of neutrophils and hematopoietic stem cells [27]. It has also shown immunomodulatory effects by generating tolerogenic DC, which induce Th2 reactions and/or regulatory T cells [28], increasing the secretion of anti-inflammatory cytokines [29]. Other functions of G-CSF include the mobilization of mesenchymal stem cells [30], which may be associated with the regeneration of mesenchymal tissues. A recent study that applied G-CSF in the culture of human cartilage fragments in a composite scaffold demonstrated outgrowing cells from the cartilage fragments, suggesting a possible phenotypic shift towards a proliferative state by G-CSF [31]. In the case of tissue-engineered cartilage, however, histological finding of the G-CSF-treated construct did not show an increase in cell number or cartilage matrix in vitro (Fig. 5C). Therefore, it was suggested that the enhanced cartilage maturation of G-CSF-treated constructs after transplantation (Fig. 6A) was mainly due to suppressed localization of macrophages by increased FasL on chondrocytes, and not due to G-CSF inducing the differentiation or proliferation of chondrocytes in in vitro culture before transplantation. Our study may propose another action of G-CSF as an inducer of FasL on chondrocytes.

Taken together, the findings of our present study suggest the following flow in the tissue reactions of tissue-engineered cartilage (Fig. 6C). Transplantation of tissue-engineered cartilage constructs initiates the infiltration of macrophages. Among the secreted cytokines from macrophages, G-CSF, IL-6 and others enhance the expression of FasL on chondrocytes, which in turn induces cell death of macrophages, suppressing tissue reactions in tissue-engineered cartilage. This series of immunological events may contribute to controlling the localization of macrophages and promote the maturation of tissue-engineered cartilage. Thus far, cartilage regenerative medicine has mainly focused on how to enhance the maturation of cartilage matrix, which we regard as a reasonable and effective approach. However, considering that the host environment is not static, or rather, transplanted cells interact with the host cells, we should pursue an additional approach to avoid detrimental tissue reactions. In our present study, we clarified the mechanisms by which chondrocytes obtain the property of immune privilege after transplantation of tissue-engineered cartilage. G-CSF, which was identified as a FasL inducer in chondrocytes, was shown to enhance the ability of chondrocytes to suppress the localization of macrophages, resulting in the promotion of cartilage maturation. This study demonstrated the efficacy of regulating hosts' reactions for cartilage regeneration, and this approach would be applicable and effective for the transplantation of other engineered tissues.

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Figure 1. Expression of FasL and Fas in tissue-engineered cartilage constructs 2 and 8 weeks after transplantation. (A) (Macro) Macroscopic images of tissue-engineered cartilage. Scale bars, 1 mm. (TB) Toluidine blue staining. Scale bars, 1 mm. (COL2, F4/80, FasL and Fas) Immunohistochemical staining for COL2, F4/80, FasL and Fas. Scale bars, 100 μm. (B) Expression of *FASL* and *FAS* in tissue-engineered cartilage was examined by real-time RT-PCR. Data are expressed as mean (bars) ± S.D. (error bars). **, $p < 0.01$, vs. 0 day.

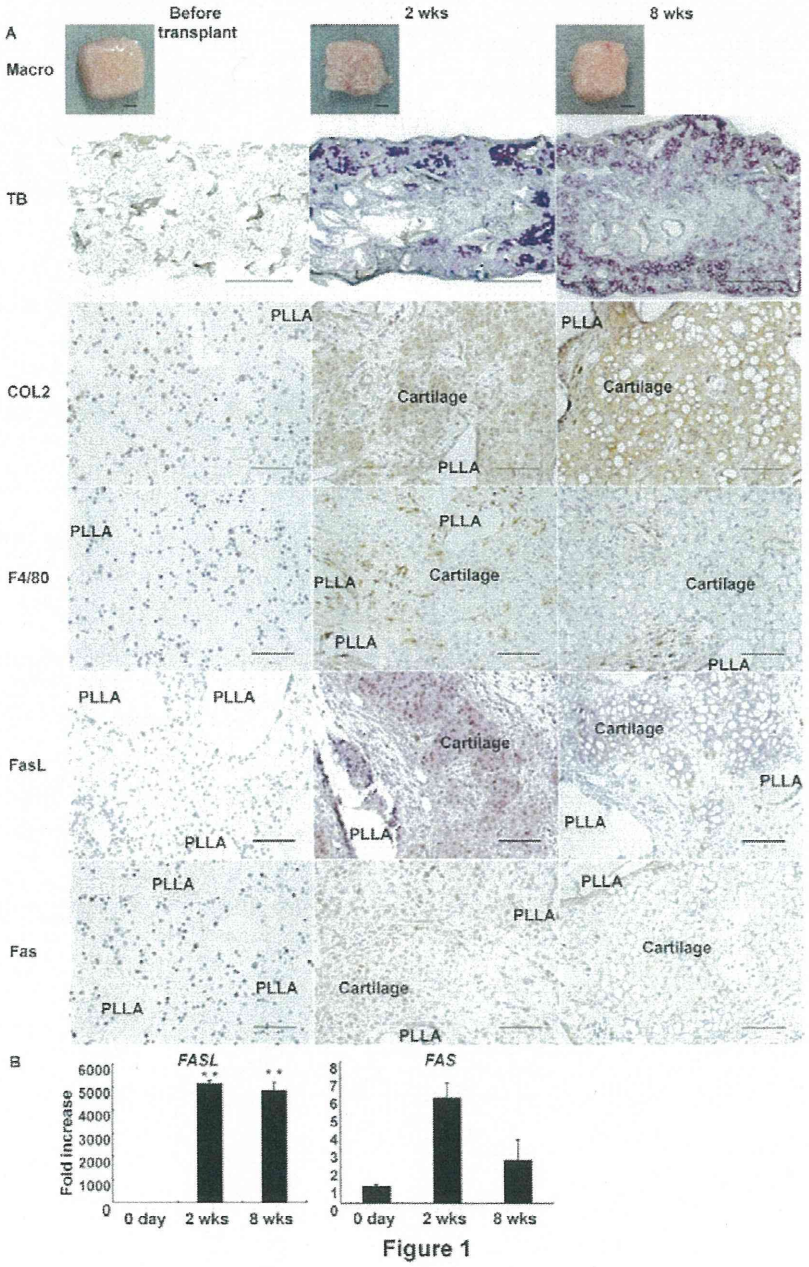

Figure 1

Figure 2. Chondrocytes hamper viability of macrophages. (A) Cell death of RAW264 (left) and activated RAW264 (right) co-cultured with wild or gld chondrocytes. Wild chondrocytes induced more cell death in RAW264 and activated-RAW264 than did gld chondrocytes. (B) Induction of apoptosis in RAW264 co-cultured with wild (left) or gld chondrocytes (right). Wild chondrocytes induced more apoptosis in RAW264 than did gld chondrocytes. (C) Cell death induced in wild or gld chondrocytes co-cultured with RAW264 (left) or activated RAW264 (right). Viability of chondrocytes was not so affected by co-cultured RAW264. (D) Human auricular chondrocytes were co-cultured with RAW264 in 3D culture, and expression of *COL2* and *COL1* in chondrocytes was examined by real-time RT-PCR. Expression of *COL2* in chondrocytes was decreased and that of *COL1* was enhanced as the ratio of RAW264 increased. Data are expressed as mean (bars) ± S.D. (error bars). *, $p < 0.05$, vs. group with 20:0 ratio. **, $p < 0.01$, vs. group with 20:0 ratio.

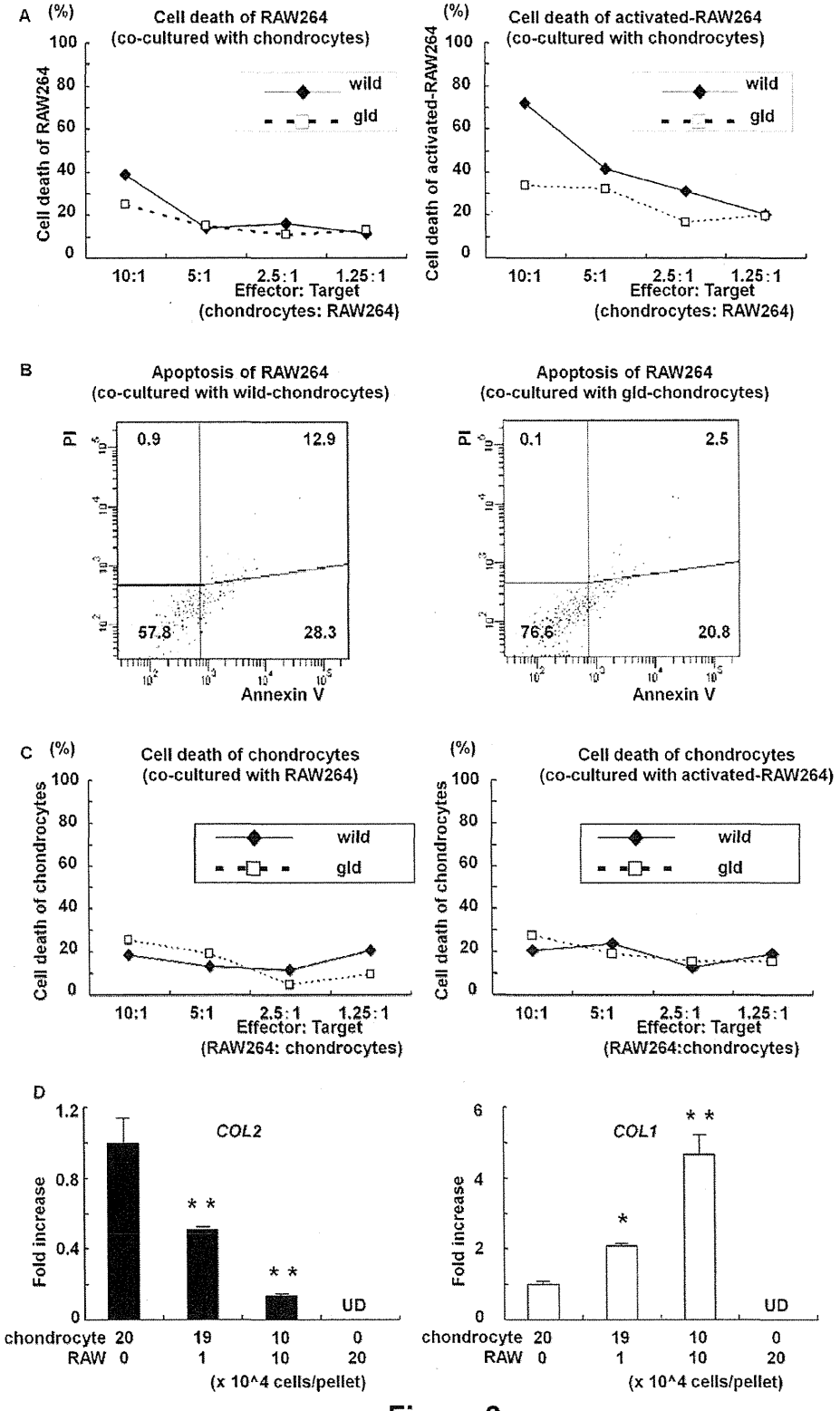
A
(%)
Cell death of RAW264
(co-cultured with chondrocytes)
Cell death of RAW264
wild
gld
Effector: Target
(chondrocytes: RAW264)
(%)
Cell death of activated-RAW264
(co-cultured with chondrocytes)
Cell death of activated-RAW264
wild
gld
Effector: Target
(chondrocytes: RAW264)
B
Apoptosis of RAW264
(co-cultured with wild-chondrocytes)
PI
0.9
12.9
57.8
28.3
Annexin V
Apoptosis of RAW264
(co-cultured with gld-chondrocytes)
PI
0.1
2.5
76.6
20.8
Annexin V
C
(%)
Cell death of chondrocytes
(co-cultured with RAW264)
Cell death of chondrocytes
wild
gld
Effector: Target
(RAW264: chondrocytes)
(%)
Cell death of chondrocytes
(co-cultured with activated-RAW264)
Cell death of chondrocytes
wild
gld
Effector: Target
(RAW264:chondrocytes)
D
COL2
Fold increase
UD
chondrocyte 20 19 10 0
RAW 0 1 10 20
(x 10^4 cells/pellet)
COL1
Fold increase
UD
chondrocyte 20 19 10 0
RAW 0 1 10 20
(x 10^4 cells/pellet)

Figure 2

Figure 3. FasL on chondrocytes promoted maturation of tissue-engineered cartilage. (A) HE staining of tissue-engineered cartilage constructs, consisting of PLLA scaffolds and auricular chondrocytes of wild or gld mice. Maturation of cartilage was suppressed in gld constructs. Scale bars, 100 μm. Toluidine blue staining (TB) of wild and gld constructs 2 and 8 weeks after transplantation. Less accumulation of extracellular matrix was noted in gld constructs. Scale bars, 500 μm. (B) GAG content of wild and gld constructs 2 and 8 weeks after transplantation. Accumulation of GAG was significantly decreased in gld constructs. Data are expressed as mean (bars) ± S.D. (error bars). *, $p < 0.05$, vs. wild.

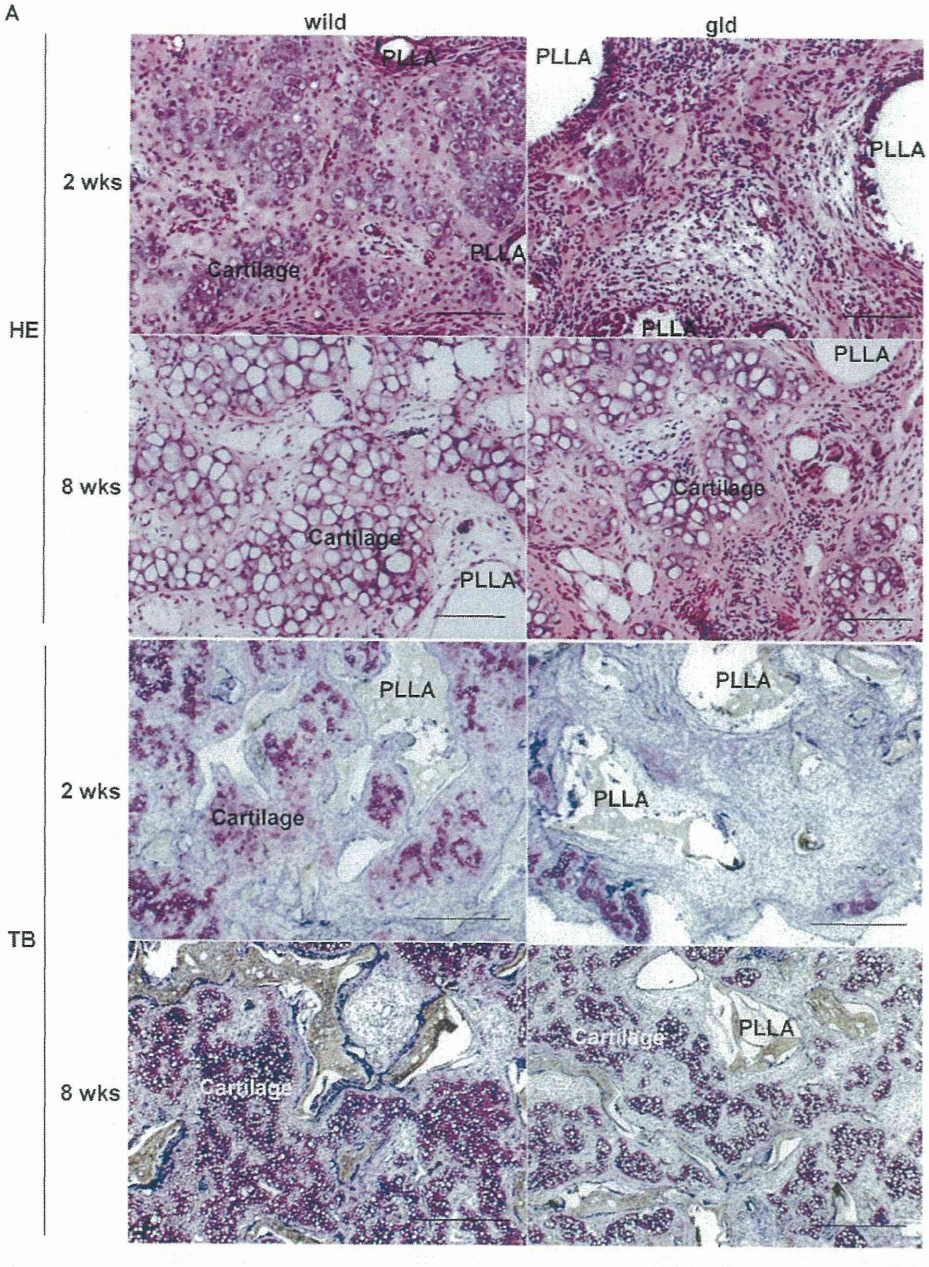
A
wild
gld
HE
2 wks
8 wks
TB
2 wks
8 wks
PLLA
Cartilage
B
(μg/mm³)
GAG content
2 wks
8 wks
wild
gld
*

Figure 3

Figure 4. FasL on chondrocytes suppressed localization of macrophages. (A) Immunohistochemical staining for F4/80 antigen and quantification of DAB-positive areas showed more localization of macrophages in gld constructs. Scale bars, 100 μm. (B) Expression of *FASL* in chondrocytes cultured alone (Chondrocyte) or co-cultured with RAW264 (Co-culture) was examined by real-time RT-PCR. Data are expressed as mean (bars) ± S.D. (error bars). **, $p < 0.01$, vs. Chondrocyte. (C) Double immunohistochemical staining for F4/80 (blue) and FasL (brown) antigen in tissue-engineered cartilage constructs 2 weeks after transplantation. Chondrocytes in the proximity of macrophages showed increased expression of FasL (arrow heads). Scale bars, 25 μm.

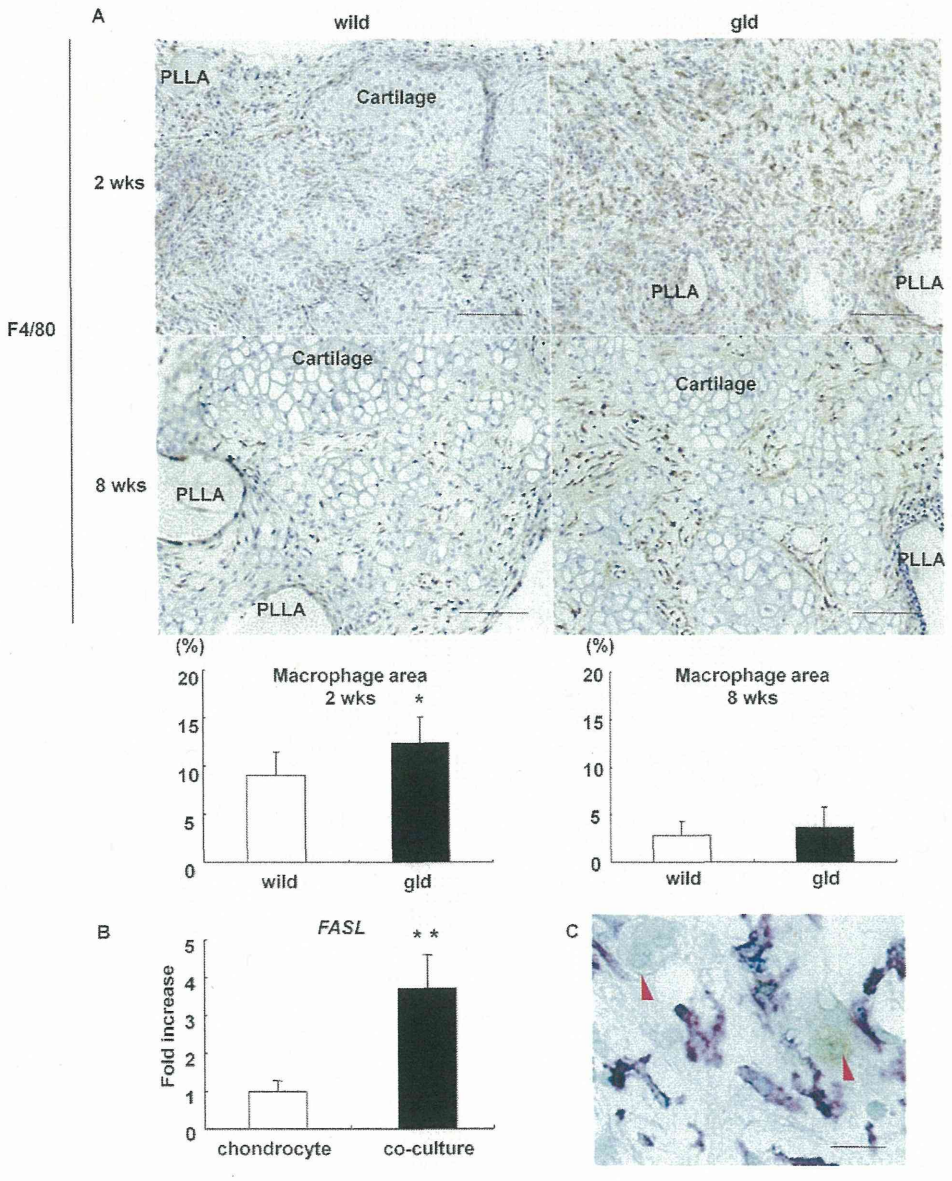

Figure 4

Figure 5. G-CSF induced FasL expression on chondrocytes. (A) A mixture of mouse auricular chondrocytes and RAW264 (chondrocytes:RAW264=19:1) or chondrocytes alone in 1% atelocollagen gel were embedded in PLLA scaffolds. After culture in re-differentiation medium for 7 days, the collected medium was used for cytokine array. Secretion of cytokines was quantified as relative ratio. (B) Human auricular chondrocytes were cultured in 3D pellets for 5 days with re-differentiation medium containing selected cytokines. The concentration of each cytokine added to the medium was ED_{50} (1) as well as one tenth ED_{50} and 10 times ED_{50} (0.1 and 10). Expression of FasL was detected when G-CSF, IL-6, KC, MIP-1α or MIP-1β was added, while it was undetectable in the control medium (Cont). (C) Immunohistochemical staining for FasL detected the expression of FasL in tissue-engineered cartilage treated with G-CSF or IL-6 for 5 days. Scale bars, 50 μm. (D) Expression of receptors for the factors identified in (B) in chondrocytes was analyzed by PCR. (E) Expression of STAT3 and p-STAT3 in human auricular chondrocytes in 3D pellets treated with or without G-CSF for 10 and 30 min. An elevated level of phospho-STAT3 was observed with the addition of G-CSF. The effect was abolished by the addition of AG490. (F) Expression of FasL in human auricular chondrocytes treated with G-CSF and AG490 for 5 days. The expression of *FASL* in chondrocytes treated with G-CSF was set as 1. Data are expressed as mean (bars) ± S.D. (error bars). *, $p < 0.05$, vs. group without AG490.