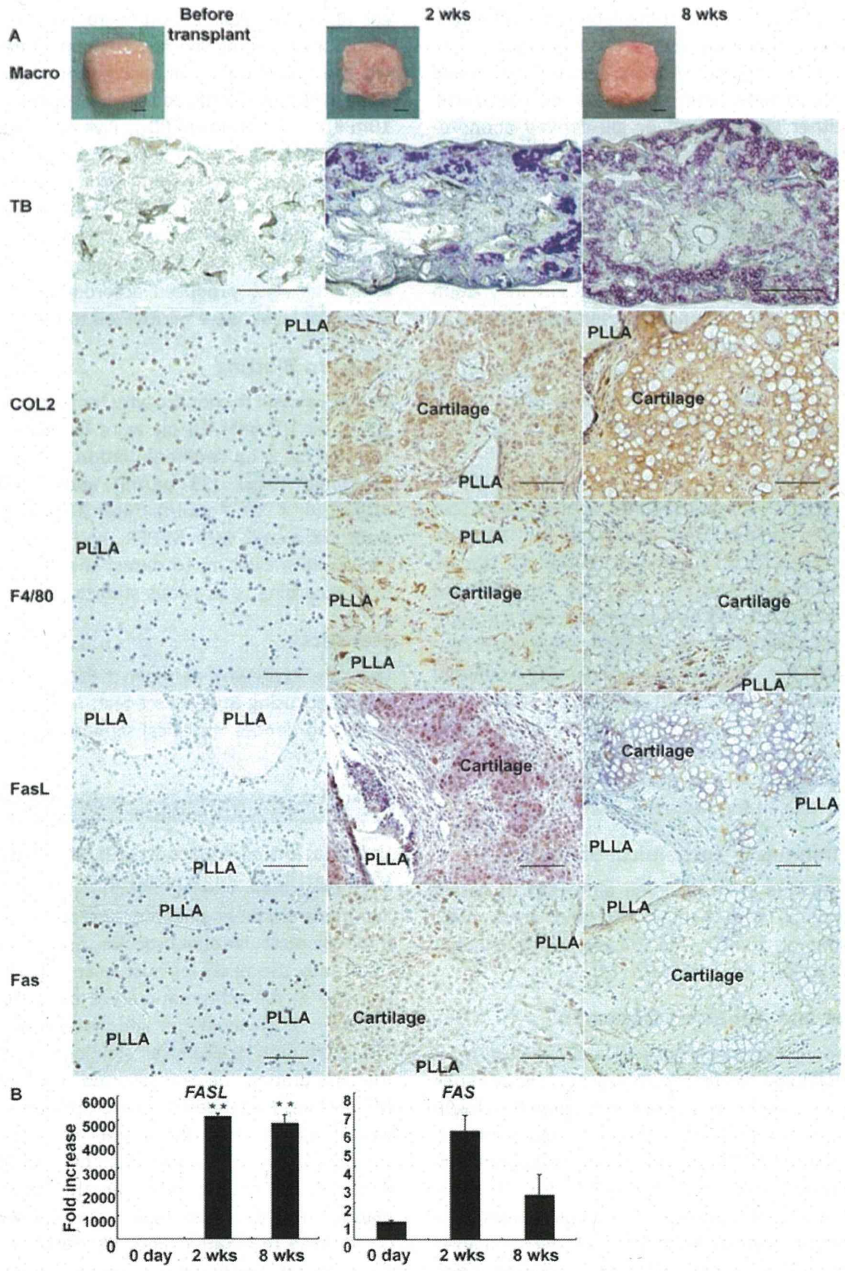

Figure 1. Expression of FasL and Fas in tissue-engineered cartilage constructs 2 and 8 weeks after transplantation. **(A):** (Macro) Macroscopic images of tissue-engineered cartilage. Scale bars = 1 mm. TB staining. Scale bars = 1 mm. (COL2, F4/80, FasL, and Fas) Immunohistochemical staining for COL2, 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) ± SD (error bars). **, $p < .01$, versus 0 day. Abbreviations: PLLA, poly(L-lactic acid); TB, toluidine blue.

hypomorphic mice, C57BL/6J-gld (gld). As macrophages have been identified as the major component of the host reaction [2], we cocultured 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 cocultured 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

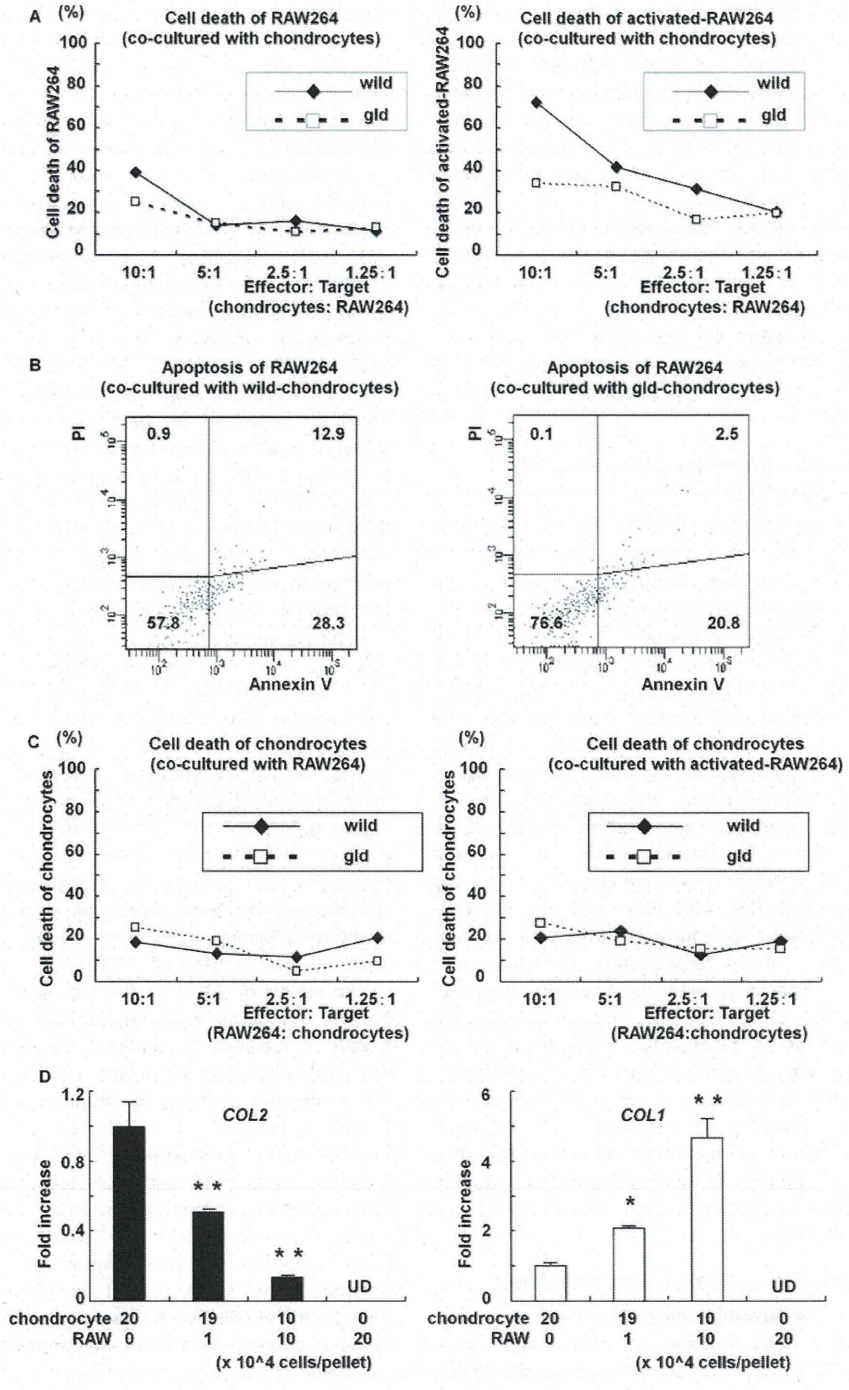

Figure 2. Chondrocytes hamper viability of macrophages. **(A):** Cell death of RAW264 (left) and activated RAW264 (right) cocultured with wild or gld chondrocytes. Wild chondrocytes induced more cell death in RAW264 and activated-RAW264 than did gld chondrocytes. **(B):** Induction of apoptosis in RAW264 cocultured 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 cocultured with RAW264 (left) or activated RAW264 (right). Viability of chondrocytes was not so affected by cocultured RAW264. **(D):** Human auricular chondrocytes were cocultured with RAW264 in three-dimensional 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) ± SD (error bars). *, $p < .05$, versus group with 20:0 ratio. **, $p < .01$, versus group with 20:0 ratio.

RAW264 in the coculture. As expected, activated RAW264 in the coculture 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 cocultured 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. H&E 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; H&E 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 three-dimensional (3D) culture of chondrocytes under differentiation stimuli [10] showed that the differentiation of chondrocytes and *FASL* expression were inversely proportional (Supporting Information Fig. S1). Next, we speculated that the enhanced expression of FasL in chondrocytes could be attributable to macrophages. Indeed, coculture 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 coculture medium of chondrocytes and RAW264 could include such molecules, we conducted a proteome array using the culture media of chondrocytes, RAW264, and cocultured chondrocytes and RAW264. In the proteome array of 40 inflammation-related cytokines, 17 cytokines were detected in the coculture 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 1/10th 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 upregulation of FasL. Indeed, human chondrocytes in 3D culture demonstrated an elevated level of phospho-STAT3 at 10 and 30 minutes 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 (Supporting Information Fig. S3A). Meanwhile, immunohistochemical staining for FasL demonstrated sustained expression of FasL even after 14 days (Supporting Information Fig. S3B), 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 nonpretreated control constructs, G-

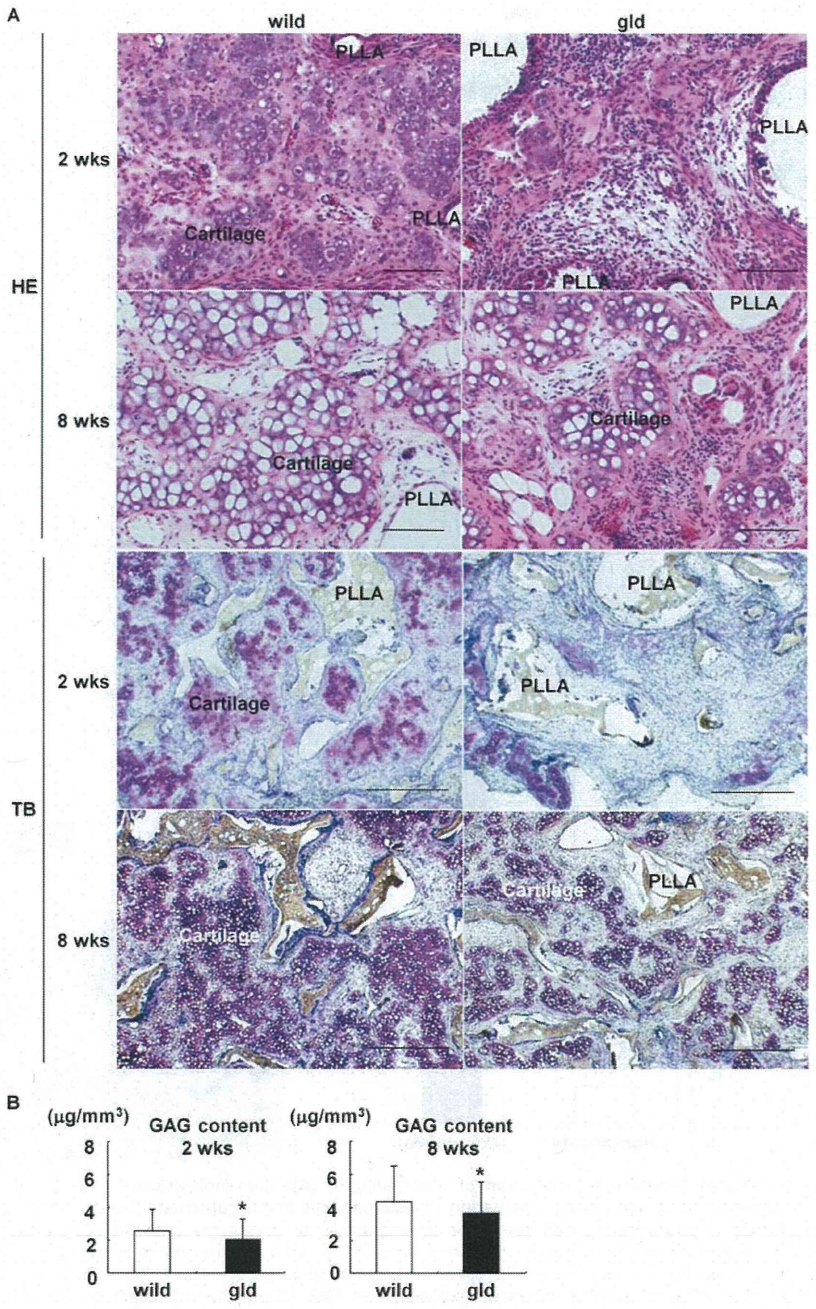

Figure 3. FasL on chondrocytes promoted maturation of tissue-engineered cartilage. **(A):** H&E staining of tissue-engineered cartilage constructs, consisting of PLLA scaffolds and auricular chondrocytes of wild or gld mice. Maturation of cartilage was suppressed in gld constructs. Scale bars = 100 μm. TB staining 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) ± SD (error bars). *, $p < .05$, versus wild. Abbreviations: H&E, hematoxylin and eosin; TB, toluidine blue.

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

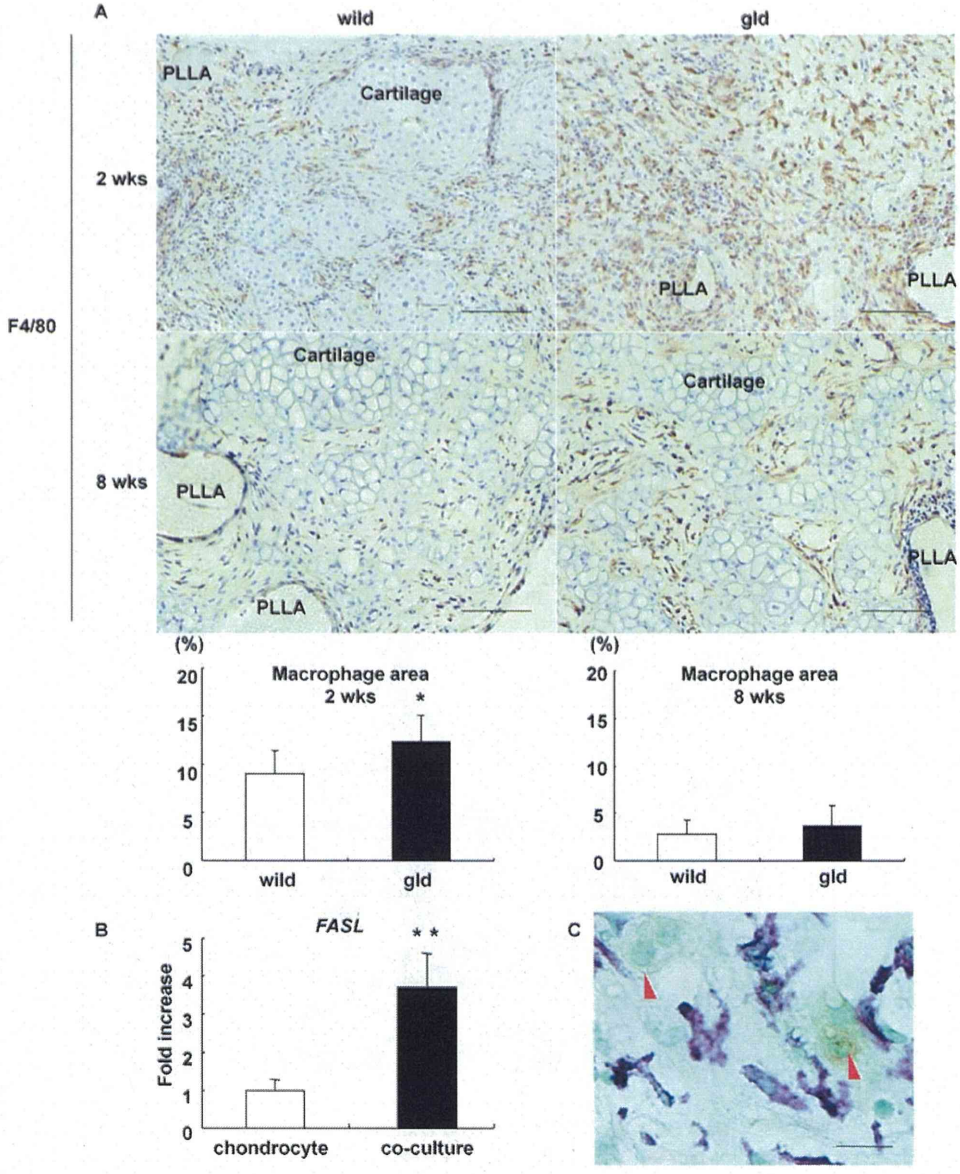

Figure 4. FasL on chondrocytes suppressed localization of macrophages. **(A):** Immunohistochemical staining for F4/80 antigen and quantification of DAB-positive areas showed more localization of macrophages in gld constructs. Scale bars = 100 μm. *, $p<.05$, versus wild. **(B):** Expression of *FASL* in chondrocytes cultured alone (chondrocyte) or cocultured with RAW264 (coculture) was examined by real-time RT-PCR. Data are expressed as mean (bars) ± SD (error bars). **, $p<.01$, versus 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. Abbreviation: PLLA, poly(L-lactic acid).

intense infiltration of macrophages than those containing wild-type chondrocytes, suggesting that FasL on chondrocytes could create an immunologically privileged environment against macrophages. Classic 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

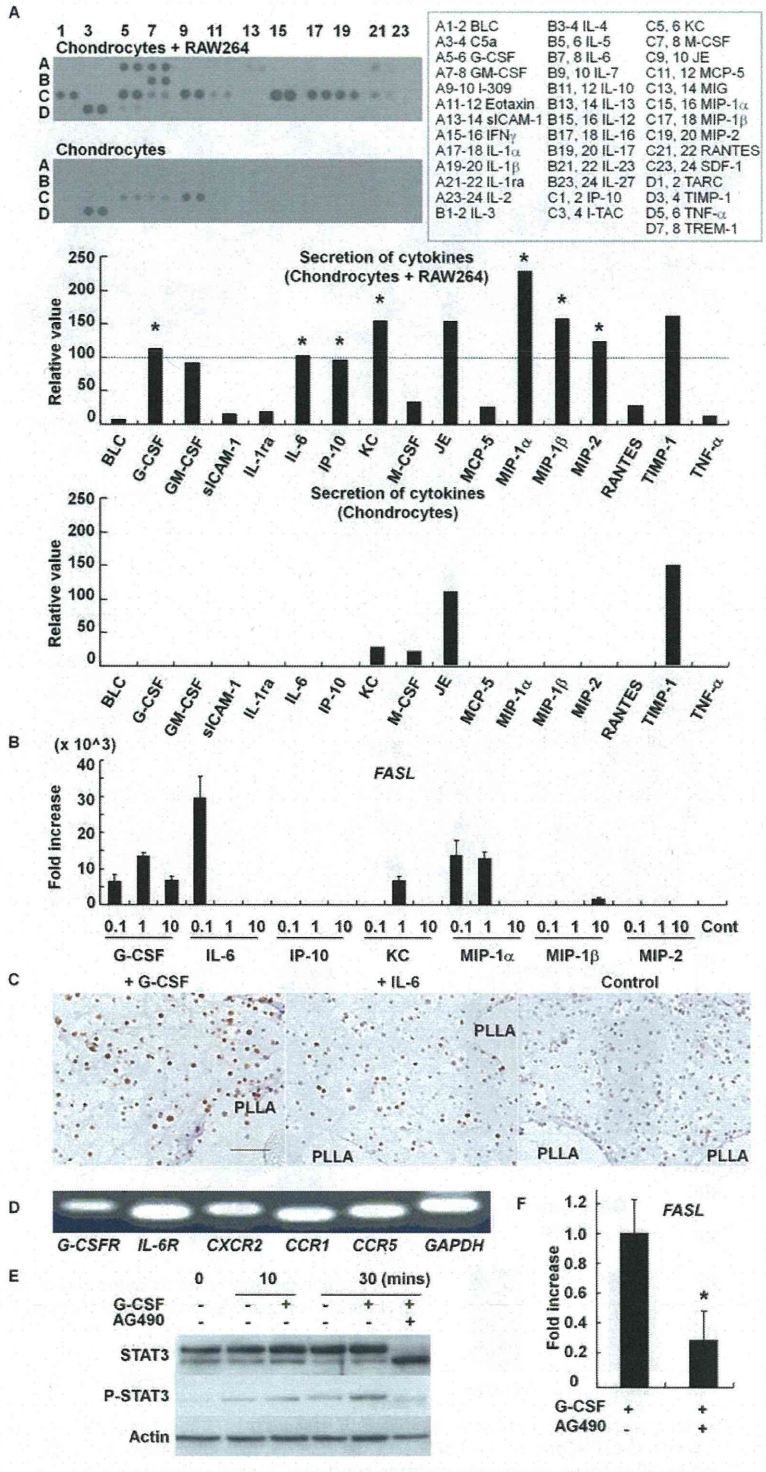

Figure 5. G-CSF induced FasL expression on chondrocytes. **(A):** A mixture of mouse auricular chondrocytes and RAW264 (chondrocytes/RAW264 = 19:1) or chondrocytes alone in 1% atelocollagen gel were embedded in PLLA scaffolds. After culture in redifferentiation medium for 7 days, the collected medium was used for cytokine array. Secretion of cytokines was quantified as relative ratio. **(B):** Human auricular chondrocytes were cultured in three-dimensional (3D) pellets for 5 days with redifferentiation medium containing selected cytokines. The concentration of each cytokine added to the medium was ED_{50} (1) as well as 1/10th 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 minutes. 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) $\pm$ SD (error bars). *, $p < .05$, versus group without AG490. Abbreviation: PLLA, poly(L-lactic acid).

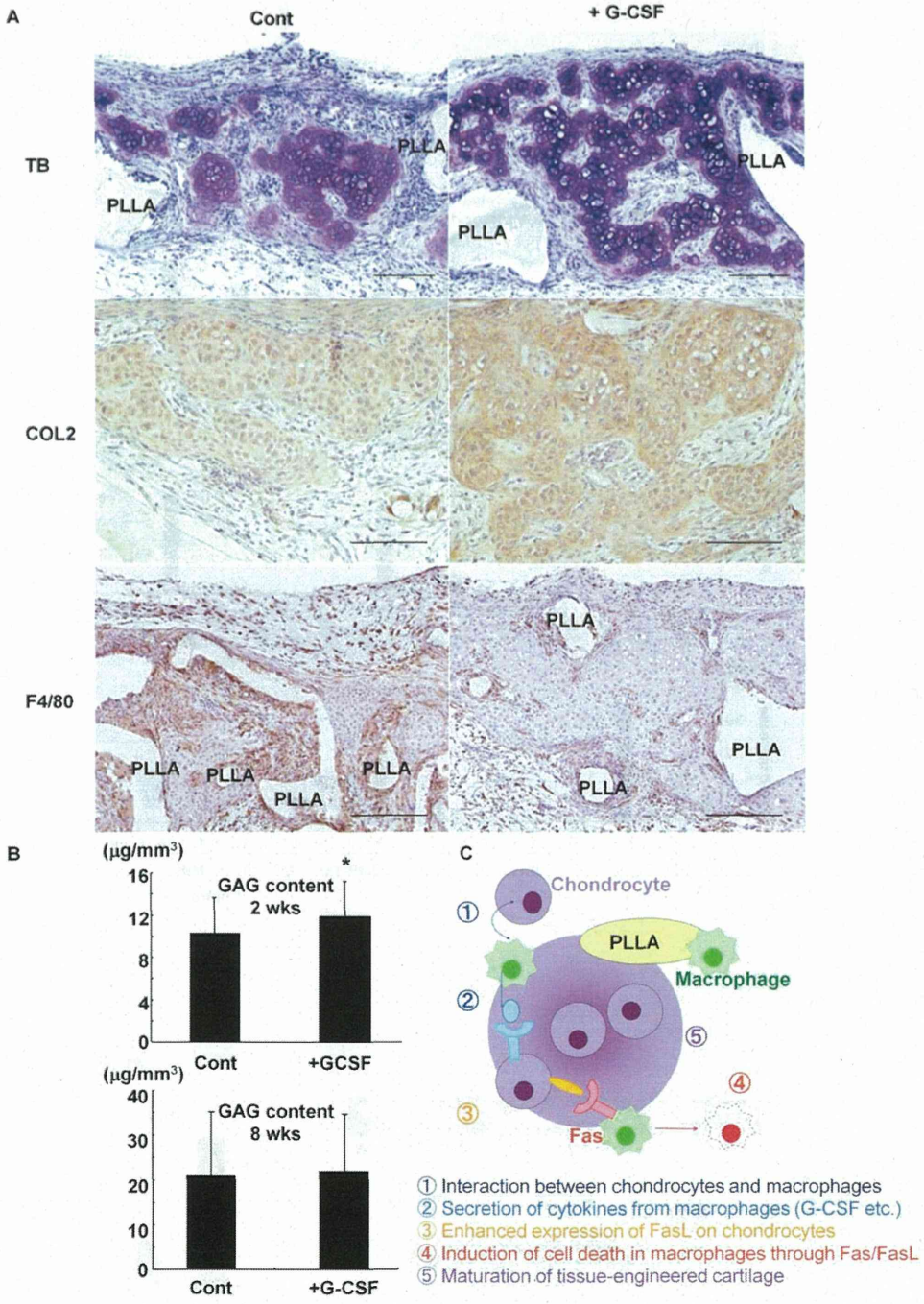

Figure 6. Application of G-CSF for transplantation of tissue-engineered cartilage. **(A):** TB staining of tissue-engineered cartilage constructs, which were treated without or with G-CSF (Cont or +G-CSF) for 5 days before transplantation. Scale bars = 50 μm. (COL2 and F4/80) Immunolocalization of COL2 and F4/80 in tissue-engineered cartilage constructs 2 weeks after transplantation, which were treated without or with G-CSF (Cont or +G-CSF) for 5 days before transplantation. Less infiltration of macrophages and enhanced accumulation of extracellular matrix were observed in G-CSF-pretreated constructs. Scale bars = 100 μm. **(B):** GAG content of tissue-engineered cartilage constructs, which were treated without or with G-CSF (Cont or +G-CSF) for 5 days before transplantation. Accumulation of GAG was significantly increased in +G-CSF constructs. Data are expressed as mean (bars) ± SD (error bars). *, $p < .05$, versus Cont. **(C):** Our theory on the formation of immune privilege in tissue-engineered cartilage. Abbreviations: PLLA, poly(L-lactic acid); TB, toluidine blue.

enzymes, complement factors, and other inflammatory cytokines, which potentially decrease the accumulation of cartilage matrix, hampering the regeneration of engineered tissues. Indeed, in this study, chondrocytes cocultured with RAW264 showed decreased expression of *COL2*, although 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 cocultured 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. 2A) indicated that activated macrophages (M1) were more susceptible to cell death than were inactivated macrophages (M2) when cocultured 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, this 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 dendritic cells, 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 toward 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 this 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.

Conclusion

In this 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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Author Contributions

Y.F.: conception and design, collection and/or assembly of data, data analysis and interpretation, and manuscript writing; T.T.: financial support, administrative support, provision of study material or patients, and data analysis and interpretation; K.H.: conception and design, financial support, administrative support, provision of study material or patients, data analysis and interpretation, manuscript writing, and final approval of manuscript.

Disclosure of Potential Conflicts of Interest

The authors indicate no potential conflicts of interest.

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宿題報告

顎顔面領域における骨・軟骨再生に関する基礎および臨床研究

Basic and clinical research on bone and cartilage regenerative medicine in the oral and maxillofacial region

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Abstract

Recently, there have been remarkable advances in regenerative medicine, and almost all disorders in the oral and maxillofacial region could be research targets of regenerative medicine. Meanwhile, treatments in this region have been well established using biomaterials, prostheses and microsurgery. Therefore, in order to be used instead of such conventional approaches, regenerative medicine should take a less invasive, more effective approach. In this report, we present our basic and clinical research on bone and cartilage regenerative medicine in the oral and maxillofacial region.

Regarding bone regenerative medicine, we have tried to develop artificial bone that maximizes bone formation at the transplanted site, but that would be subsequently replaced by autologous bone. We have made custom-made artificial bone (CT-Bone) using α-TCP particles and an ink-jet printer, and have conducted clinical research and trials on 30 patients.

Present protocols of autologous chondrocyte transplantation mostly utilize chondrocytes suspended in solution or gel, and thus the regenerated cartilage may fail biologically and biophysically. To develop tissue-engineered cartilage with proper 3D morphology and mechanical strength, we have optimized the culture medium of chondrocytes and scaffold. Following a preclinical study confirming efficacy and safety, our protocol has been approved by the Institutional Review Board as well as the Ministry of Health, Labour and Welfare, Japan. We have conducted clinical research for 3 patients with nasal deformity in cleft lip and palate, and are now starting multicenter clinical research. Also, we are preparing for investigator-initiated clinical trials of tissue-engineered trachea for reconstruction of trachea.

Key words：bone and cartilage regenerative medicine（骨軟骨再生医療），oral and maxillofacial region（顎顔面領域），CT-Bone，implant-type tissue-engineered cartilage（インプラント型再生軟骨）

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