Genome Engineering of Stem Cells for Autonomously Regulated, Closed-Loop Delivery of Biologic Drugs

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SUMMARY

Chronic inflammatory diseases such as arthritis are characterized by dysregulated responses to pro-inflammatory cytokines such as interleukin-1 (IL-1) and tumor necrosis factor α (TNF-α). Pharmacologic anti-cytokine therapies are often effective at diminishing this inflammatory response but have significant side effects and are used at high, constant doses that do not reflect the dynamic nature of disease activity. Using the CRISPR/Cas9 genome-engineering system, we created stem cells that antagonize IL-1- or TNF-α-mediated inflammation in an autoregulated, feedback-controlled manner. Our results show that genome engineering can be used successfully to rewire endogenous cell circuits to allow for prescribed input/output relationships between inflammatory mediators and their antagonists, providing a foundation for cell-based drug delivery or cell-based vaccines via a rapidly responsive, autoregulated system. The customization of intrinsic cellular signaling pathways in stem cells, as demonstrated here, opens innovative possibilities for safer and more effective therapeutic approaches for a wide variety of diseases.

INTRODUCTION

Chronic inflammatory and autoimmune diseases such as arthritis are characterized by aberrant activity of cytokines such as tumor necrosis factor α (TNF-α) and interleukin-1 (IL-1). These pro-inflammatory mediators are expressed by a variety of cells, including synovial cells, osteoblasts, myotubes, satellite cells, chondrocytes, and innate immune cells. These cell types are also capable of responding to TNF-α and IL-1 through canonical signaling via cognate cell surface receptors. Under normal physiologic conditions, appropriate signaling of TNF-α and IL-1 contributes to organ and tissue homeostasis by promoting tissue remodeling (Banno et al., 2004; Furman et al., 2014; Gerstenfeld et al., 2003; Kimmerling et al., 2015; Peralta Soler et al., 1996), orchestrating phagocytosis of cellular debris and immunogenic substrates (Michlewskà et al., 2009), and coordinating transitions between niche stem cell quiescence and proliferation/differentiation programs (Palacios et al., 2010; Rebel et al., 1999; Rezzoug et al., 2008). However, in chronic diseases, elevated levels of these pro-inflammatory cytokines can lead directly to pain (Marchand et al., 2005), cytotoxicity (Georgopoulos et al., 1996), accelerated tissue catabolism (Bonaldo and Sandri, 2013; Reid and Li, 2001), and exhaustion of resident stem cell niches (Palacios et al., 2010).

A number of exogenous anti-cytokine therapies have been shown to effectively counteract the negative sequelae of TNF-α and IL-1 dysregulation. In particular, anti-TNF therapies such as the soluble type 2 TNF receptor (etanercept) and monoclonal antibodies to TNF-α (adalimumab, infliximab) have demonstrated efficacy toward offsetting pain associated with chronic and rheumatic diseases, including arthritis (Scott and Kingsley, 2006). The soluble type 1 TNFR receptor (sTNFR1) has also been investigated as a gene therapy for treatment of chronic diseases (Khoury et al., 2007). More recently, competitive antagonists of IL-1 such as IL-1 receptor antagonist (IL-1Ra, anakinra) have been shown to alleviate symptoms of rheumatoid arthritis (Choy et al., 2013) and the onset of post-traumatic arthritis (Furman et al., 2014). Although they are effective, these therapies are administered at very high and generally unregulated doses. Due to the pleiotropic roles of TNF-α and IL-1 and their involvement in tissue homeostasis, the use of such therapies may have significant side effects, including increased susceptibility to infection as well as to autoimmune diseases such as lupus, interstitial lung disease, and vasculitis (Ramos-Casals et al., 2008). Moreover, excess inhibition of these cytokines can interfere with tissue regeneration and repair (Gopinath and Rando, 2008; Kimmerling et al., 2015; Mozzetta et al., 2009; Palacios et al., 2010). Therefore, methods to dynamically deliver precisely calibrated doses of anti-inflammatory biologic therapies could improve treatments by combating cytokine-mediated pain and degeneration while spatially and...
temporally regulating the production of anti-cytokine drugs.

Here, we propose a regenerative medicine approach to the treatment of chronic inflammatory diseases by engineering cells that execute real-time, programmed responses to environmental cues, including pro-inflammatory cytokines. We used genome editing with the CRISPR/Cas9 system to create stem cells that antagonize IL-1- and TNF-α-mediated inflammation in an autoregulated manner. To achieve this, we selected to overtake the chemokine (C-C ligand 2 (Ccl2) gene, which is also known as macrophage chemoattractant protein-1 (Mcp-1). The Ccl2 gene product regulates trafficking of monocytes/macrophages, basophils, and T lymphocytes (Ping et al., 1999). TNF-α and IL-1 serve as two of the most potent stimulators of Ccl2 expression (Boekhoudt et al., 2003); however, the persistence of Ccl2 expression depends on continued exposure to inflammatory cues (Hao and Baltimore, 2009), so resolution of inflammation results in rapid decay of Ccl2 transcripts. Thus, we performed targeted gene addition of IL-1- and TNF-α antagonists at the Ccl2 locus to confer cytokine-activated and feedback-controlled expression of biologic therapies. These programmed stem cells were then used to engineer articular cartilage tissue to establish the efficacy of self-regulated therapy toward protection of tissues against cytokine-induced degeneration. We hypothesized that this approach of repurposing normally inflammatory signaling pathways would allow for transient, autoregulated production of cytokine antagonists in direct response to cytokine stimulation. This type of approach could provide an effective “vaccine” for the treatment of chronic diseases while overcoming limitations associated with delivery of large drug doses or constitutive overexpression of biologic therapies.

RESULTS

Clonal Isolation and Functional Validation
The primary goal of this work was to program induced pluripotent stem cells (iPSCs) with the capacity to respond to an inflammatory stimulus with potent and autonomously regulated anti-cytokine production (Figure 1A). As such, we aimed to perform targeted gene addition to the locus of the pro-inflammatory chemokine Ccl2, which is potently activated in response to cytokine-mediated signaling and of which disruption of a single allele would not be expected to compromise overall cellular function. Thus, transgenes encoding a firefly luciferase transcriptional reporter or a cytokine antagonist, either murine IL-1Ra or a chimeric human sTNFR1-murine immunoglobulin G (Bloquel et al., 2004), were targeted to the Ccl2 start codon in murine iPSCs (Diekman et al., 2012) using the CRISPR/Cas9 gene-editing platform. After hygromycin selection, clonal isolation, and screening by PCR of the junctions of the transgene and target locus, multiple clones were identified that possessed targeted integration events at the Ccl2 locus (Figure S1).

Clones for each transgene with targeted gene addition on one allele were selected for further analysis (referred to as Ccl2-Luc, Ccl2-Il1ra, or Ccl2-sTNFR1) and expanded on murine embryonic fibroblasts (MEFs) followed by pre-differentiation in micromass culture (Diekman et al., 2012). First, we evaluated whether targeted transgene integration at the Ccl2 start codon would enable cytokine-inducible transgene expression. As a point of reference, wild-type (WT) cells were treated with a range of TNF-α concentrations (0.2–20 ng/mL), and mRNA was collected at 4, 12, 24, and 72 hr (Figure 1B). Ccl2 gene expression was evaluated by qRT-PCR. At all TNF-α concentrations tested, Ccl2 gene expression was elevated at each time point compared with cells cultured in the absence of TNF-α (p < 0.016). In the 2-ng/mL and 20-ng/mL groups, Ccl2 gene expression continued to increase throughout the 72-hr period of TNF-α treatment (p < 1.8e-10).

Next, using two Ccl2-luciferase cell lines, we induced luciferase expression by stimulating cells with 20 ng/mL TNF-α to evaluate whether transgene expression reflected endogenous Ccl2 expression in WT cells. Relative luminescence measurements indicated that transgene expression in both clones was indeed stimulated by cytokine and increased across the 72-hr TNF-α treatment period (p < 8.5e-10, Figure 1C), consistent with findings from TNF-induced Ccl2 expression in WT cells.

Dynamic, Feedback-Controlled Biologic Drug Production in Stem Cells
We then probed the responsiveness of our engineered cells endowed with Ccl2-driven anti-cytokine transgenes. We performed these experiments primarily by evaluating gene expression and transgene production in the Ccl2-sTNFR1 group, as the inability of these murine cells to otherwise produce this human transcript and protein allows for direct conclusions regarding transgene production from the Ccl2 locus.

Initially, we performed a time-course and dose-response experiment, in which Ccl2-sTNFR1 and WT cells were treated with a range of TNF-α concentrations (0.2–20 ng/mL) for a variety of times (4, 12, 24, and 72 hr). We measured the expression of the sTNFR1 transgene at both the mRNA and protein levels by qRT-PCR and ELISA, respectively. We also measured the expression of Il6, a pro-inflammatory cytokine whose expression serves as a sentinel marker of inflammation, at the mRNA level by qRT-PCR in order to additionally characterize the inflammatory response of the WT and engineered Ccl2-sTNFR1 cells.
As early as 4 hr after TNF-α treatment, the 2- and 20-ng/mL treatments significantly upregulated Il6 transcription in both the WT and Ccl2-sTNFR1 cells, while 0.2 ng/mL did not significantly upregulate Il6 (Figure 2A). At the 12-hr time point, Il6 expression was significantly elevated at all TNF-α concentrations in WT cells; however, Il6 was only significantly upregulated in the Ccl2-sTNFR1-engineered cells at the 20-ng/mL level of TNF-α treatment (Figure 2A). Even at the 20-ng/mL level of treatment, the engineered cells showed a significantly lower level of Il6 induction than WT cells. At the 24-hr time point, the medium and high concentrations of TNF-α drove an upregulation of Il6 in WT cells, but only the high 20-ng/mL concentration resulted in significant upregulation of Il6 in the sTNFR1-engineered cells (Figure 2A). By the 72-hr time point, all three doses of TNF-α resulted in significant upregulation of Il6 in the WT cells, while TNF-α treatment only induced an upregulation of Il6 in the Ccl2-sTNFR1 cells at the 20-ng/mL treatment level (Figure 2A). These results show reduced inflammatory response as a result of cytokine-mediated induction of sTNFR1 from the Ccl2 locus.

To evaluate whether the observations of Il6 gene expression reflect the general state of inflammation in these cells, we transduced WT and Ccl2-sTNFR1 cells with a lentiviral vector delivering a nuclear factor κB (NF-κB) luminescence reporter. We then treated these cells with 0 or 20 ng/mL TNF-α and after 24, 48, and 72 hr measured luminescence as a surrogate for activity of the NF-κB transcription factor.

Figure 1. Depiction of the Reprogrammed Inflammatory Signaling Pathway in CRISPR/Cas9-Engineered Cells and Results Validating the Approach

(A) Top left: in wild-type (WT) cells, TNF-α signaling through its type 1 receptor initiates a cascade leading to nuclear translocation and increased transcriptional activity of NF-κB, activating an inflammatory transcriptional program. One gene rapidly and highly upregulated by cytokine-induced NF-κB activity is Ccl2 (shown in orange). Top right: a CRISPR/Cas9 RNA-guided nuclease (not depicted) generates a double-strand break in the endogenous chromosomal locus near the start codon for Ccl2. Provision of a targeting vector with a transgene flanked by regions homologous to the Ccl2 locus promotes the use of this template for repair of the damaged allele in a subset of cells. Bottom left: such alleles would then be activated by TNF-α, which would now induce expression of the soluble TNF type 1 receptor (sTNFR1). Bottom right: upon antagonism of TNF-α in the microenvironment, signal transduction through the membrane receptor would halt, NF-κB would remain sequestered in the cytoplasm, and expression of the sTNFR1 transgene would autonomously decay upon resolution of the local inflammation.

(B) qRT-PCR data showing the expression profile of Ccl2 after treatment of WT cells with various concentrations of TNF-α (n = 3 independent experiments). Values plotted represent the mean fold change in expression ± SEM compared with untreated controls of each cell line. *p < 0.05 between each time point for each clone, and also *p < 0.05 between clones for each time point. See also Figure S1 and the appended table.
which is activated in response to various inflammatory signals. At 24 hr, the NF-κB transcriptional activity was upregulated in both WT and Ccl2-sTNFR1 cells. However, at the 48- and 72-hr time points, a sharp decline in NF-κB transcriptional activity was observed in engineered cells expressing sTNFR1 under control of the Ccl2 locus (Figure 2B).

Taken together, the Il6 gene expression and NF-κB transcriptional assays further support that the Ccl2-sTNFR1 cells are capable of attenuating the TNF-α-induced regulation of Il6 as well as a more general inflammatory state. Furthermore, these results suggest that, after 3 days of TNF-α treatment, the cells are capable of antagonizing even a high (20 ng/mL) concentration of TNF-α and as normalized by the r18S reference gene. The 0-hr time point (shaded) was not measured and is shown for illustration purposes only, as all samples at 0 hr measure 1 by definition.

We then performed iterative stimulation of Ccl2-driven sTNFR1 and IL-1Ra cells in monolayer with either 0.1 ng/mL IL-1α or 20 ng/mL TNF-α. After 24 hr, the cytokine-containing medium was exchanged for cytokine-free medium, and specimens were collected. Three days later, cells were stimulated with cytokine again to establish the capacity of the cells to respond to recurrent stimulation with cytokine. Control specimens without cytokine stimulation were maintained in parallel. sTNFR1-engineered cells displayed a basal level of production of less than 3 ng/mL (Figures 3A and 3B).
produced sTNFR1 after either IL-1 or TNF-α stimulation (Figures 3A and 3B). Withdrawal of cytokine-containing medium resulted in a decline in sTNFR1 accumulation over subsequent collection periods, irrespective of whether IL-1 or TNF-α served as the stimulant. In both cases, production of sTNFR1 decreased to basal levels within 48 hr of removing cytokines (Figure 3A).

When treated with 0.1 ng/mL IL-1, which sTNFR1 should not antagonize, there was approximately 300-fold stimulation of sTNFR1 production (Figure 3B) to ~630 ng/mL. When treated with 20 ng/mL TNF-α, production of sTNFR1 increased only approximately 50-fold over basal levels to ~90 ng/mL. Similarly, treatment of Ccl2-Il1ra cells with IL-1 resulted in an increase of IL-1Ra protein in the medium of approximately 30-fold over basal levels of expression to ~180 ng/mL, whereas treatment with TNF-α resulted in an increase of approximately 88-fold to ~570 ng/mL (Figure 3C). In WT cells, IL-1Ra production after a single pulse was 1.65 ± 0.35, 1.74 ± 0.11, and 1.84 ± 0.16 ng/mL after treatment with no cytokine, 0.1 ng/mL IL-1, or 20 ng/mL TNF-α, respectively (Figure S2). Thus, in the case of both Ccl2-Il1ra and Ccl2-sTNFR1 cells, either IL-1 or TNF-α was capable of potently inducing transgene expression. However, a lower level of induction was achieved when an antagonizing therapy was produced in response to the stimulatory cytokine.

**Autoregulated Production of Cytokine Antagonists Protects Engineered Cartilage from IL-1- and TNF-Mediated Catabolism**

After establishing that engineered cells express transgenes in a cytokine-inducible manner and that Ccl2-driven sTNFR1 provides a tunable and effective response to even a high dose of TNF-α in monolayer experiments, we assessed whether tissues engineered from engineered stem cells could overcome the degenerative effects of TNF-α and IL-1. To this end, we further differentiated the WT, Ccl2-Luc, Ccl2-Il1ra, and Ccl2-sTNFR1 cells toward the chondrocyte lineage for the production of engineered cartilage tissue (Diekman et al., 2012). Engineered tissues from WT and Ccl2-Luc cell lines were treated with 0 ng/mL cytokine, 0.1–1 ng/mL IL-1, or 20 ng/mL TNF-α. Engineered tissues from Ccl2-Il1ra and Ccl2-sTNFR1 cell lines were treated with only IL-1 or TNF-α, respectively, at the same concentrations as WT and Ccl2-Luc tissues.

Engineered cartilage specimens derived from WT and Luc cell lines exhibited a significant degradative response to this 72-hr cytokine treatment. We measured the changes in gene expression induced by 1 ng/mL IL-1 or 20 ng/mL TNF-α by qRT-PCR (Figures 4 and 5, respectively) and observed significant upregulation of markers of inflammation, such as Ccl2 and Il6, as well as degradative enzymes, such as matrix metalloproteinases and aggrecanases. Furthermore, significant suppression of expression of matrix components of cartilage, including collagen type 2 α1 (Col2a1) and aggrecan (Acan), was noted in cartilage engineered from either WT or Ccl2-Luc cells. The cartilage derived from these control cell lines also displayed a loss of sulfated glycosaminoglycan (sGAG), a major component of articular cartilage critical to proper tissue function, in response to both concentrations of IL-1 and to 20 ng/mL TNF-α (Figures 6A–6D).

Cartilage derived from Ccl2-Il1ra or Ccl2-sTNFR1 cells displayed a markedly different response to cytokine treatment at the gene expression level. Tissue generated from both the Ccl2-Il1ra and Ccl2-sTNFR1 cell lines demonstrated lower induction levels of inflammatory and degradative gene products compared with cartilage engineered...
from WT or Ccl2-Luc cell lines (Figures 4 and S, respectively). Although haploinsufficiency of the Ccl2 gene affected basal levels of Ccl2 transcripts in the Ccl2-Luc, Ccl2-sTNFR1, and Ccl2-Il1ra cells compared with WT cells, cytokine stimulation rendered more marked upregulation of Ccl2 in cartilage derived from the Luc cells than sTNFR1 or Il1ra cells at 72 hr, suggesting that these transgenes ameliorated the impact of cytokine on Ccl2 gene expression, as expected. It is noteworthy, however, that in some cases these genes were still significantly upregulated relative to tissues treated with 0 ng/mL cytokine. In the case of Ccl2-sTNFR1, cartilage aggregates displayed resilience after 72 hr of treatment with TNF-α, with no suppression of Col2a1 or Acan. The preservation of a more homeostatic gene expression profile was consistent with the biochemical composition of cartilage aggregates engineered from the Ccl2-sTNFR1 cell line, which demonstrated preservation of sGAG in the tissue even after treatment with 20 ng/mL TNF-α, as determined by both biochemical and histologic analyses (Figures 6A and 6E). TNF-α induced secretion of sTNFR1, as specimens treated with 20 ng/mL TNF-α produced 18.45 ± 0.17 ng/mL sTNFR1 and those cultured in the absence of TNF-α produced only 3.31 ± 0.17 ng/mL sTNFR1.

However, Ccl2-driven expression of IL-1Ra was not sufficient to protect against the suppression of the extracellular matrix constituents Col2a1 and Acan by 1 ng/mL IL-1. Coupled with the increased expression of degradative enzymes, this resulted in loss of a significant fraction of sGAG in the engineered tissue (Figure 6B). At the 0.1 ng/mL IL-1 level, cartilage derived from engineered Ccl2-II1ra cells was less susceptible to degradation than tissue derived from control Ccl2-Luc cells, although sGAG loss normalized to total DNA content was still statistically significant after cytokine treatment in both tissue types (Figures 6C and 6D). This protection, in comparison with cartilage derived from Ccl2-Luc cells, was imparted by the cytokine-induced expression of 20.50 ± 0.67 ng/mL IL-1Ra, which was higher than the basal expression of 1.82 ± 0.24 ng/mL observed in the engineered cells or 0.88 ± 0.25 ng/mL observed in Ccl2-Luc cells.

**DISCUSSION**

Overcoming aberrant pro-inflammatory signals in chronic diseases while preserving critical homeostatic signaling nodes represents a significant challenge for regenerative medicine. This work demonstrates the utility of genome editing for the development of “designer” stem cells that sense levels of inflammation and respond according to the degree of the pathology. Using CRISPR/Cas9, we engineered pluripotent stem cells with the prescribed feature of inflammatory cytokine resistance by performing
targeted addition of therapeutic transgenes to the cytokine-responsive Ccl2 locus. Transgene expression from engineered cells was feedback-controlled with rapid on/off dynamics and was adequate to mitigate the inflammatory effects of physiologic concentrations of both IL-1 and TNF-α in the context of precursor cells cultured in monolayer as well as in engineered tissues such as cartilage. These cells provide the foundation for a cell-based vaccine for the treatment of a variety of autoimmune or inflammatory diseases.

In this work, we sought to commandeering an endogenous gene promoter to engineer custom-designed stem cells with the ability to regulate anti-cytokine therapy in an autonomous, real-time fashion. Critical to our selection of Ccl2 as the target locus for controlling transgene expression is the temporal pattern associated with its cytokine-inducible expression profile. By targeting our transgenes to the Ccl2 start codon, we preserved many of the endogenous regulatory features associated with Ccl2 expression, including distal and proximal regulatory regions encompassing two NF-κB regulatory elements as well as SP1 and AP-1 binding sites (Ping et al., 1999; Teferedegne et al., 2006; Wang et al., 2000). As such, the repurposed Ccl2 promoter did indeed endow engineered cells with the capacity to substantially upregulate transgene expression in an inflammation-inducible manner. Importantly, this upregulation was both dose- and time-dependent and was transient in nature. Treatment with a range of TNF-α concentrations spanning three orders of magnitude resulted in differential induction of transgene transcription. The concomitant decay in transgene expression and transcription of markers of inflammation such as Il6 suggests that cells were capable of autonomously tuning expression of the transgene. Importantly, our experiments also demonstrated that cells continue to respond to cytokines by robustly producing additional therapy after iterative exposure. Insertion of our transgene cassette did, however, uncouple regulation of our system from the endogenous AU-rich elements in the 3’ UTR of Ccl2, which are thought to play a role in driving transcript levels back toward a basal state after inflammation is resolved (Hao and Baltimore, 2009). Despite this, expression of our transgenes did decay after resolution of cytokine stimulation, which came about by transgene therapy or simple withdrawal of cytokine. In future iterations of this work, preservation of the AU-rich elements in the transgene cassette may provide a means whereby even more rapid declines in transgene expression may be achieved.

Basal levels of transgene product were detected in the absence of cytokine by ELISA. This observation is not surprising, as Ccl2 is detected at the protein level from certain tissues without activation from cytokines.
Despite this, even low levels of cytokine treatment were capable of inducing transgene expression and initiating the inflammatory transcriptional program, suggesting that basal levels of cytokine antagonists were insufficient to abolish signaling from low concentrations of cytokine. Concentrations as low as 2–6 pg/mL (Li and Schwartz, 2001; Li, 2003) of TNF-α are important for proper muscle regeneration and repair. Thus, since the engineered cells are capable of responding to low levels of cytokine, the basal levels of anti-cytokine therapy may not preclude detection and response of niche cells to low but critical levels of cytokine.

Our data reveal apparent differences in cell responses to inflammatory cytokines depending on their differentiation status or lineage commitment. We attribute this discrepancy to potential differences in cell number, differentiation state of the cells, and natural variation in gene expression profiles of cells adopting different phenotypes. These observations could also be strictly related to the physical features of the different culture systems. Specifically, the effective concentration of TNF-α or IL-1 could be reduced by one to two orders of magnitude due to partitioning within a tissue matrix. Moreover, secreted sTNFR1 and IL-1Ra may remain bound by the rich extracellular matrix in the engineered cartilage, whereas factors secreted by cells in monolayer remain more readily accessible to diffusion in culture media.

Previous investigators have taken various approaches to confer inflammation-inducible, autoregulatory features to target cells. A prevailing strategy has involved cloning approximately 3 kb of characterized, cytokine-inducible promoters upstream of transgene coding sequences. One example of this strategy is the use of the E-selectin promoter (Garaulet et al., 2013). In other studies, a self-limiting promoter construct was developed based on a truncated promoter sequence of cyclooxygenase-2 upstream of the IL-4 gene to express IL-4 only in the presence of inflammation (Rachakonda et al., 2008). Alternatively, tandem repeats of NF-κB response elements have been used to drive transgene expression (Khoury et al., 2007; van de Loo et al., 2004). These expression cassettes are typically delivered to cells by viral gene delivery. While these approaches have indeed been proved to be effective at generating cytokine-inducible expression, notable limitations do exist. Most of these relate to the reliance on viral vectors to deliver the expression cassette and include the limited packaging capacity of adeno-associated virus, which restricts the size of the cloned promoter sequence.
and the potential for insertional mutagenesis by lentiviral vectors. Furthermore, reported basal levels of transgenes produced from these promoters have been high, and, in some cases, growth factors other than inflammatory mediators are needed to co-stimulate efficient induction of transgene expression (Garaulet et al., 2013). While such gene delivery vehicles carrying inflammation-inducible cassettes could be delivered in vivo, this method lacks cell- and tissue-targeting specificity.

Our work extends these efforts by directly targeting transgenes to inducible, endogenous loci using the efficient and highly specific CRISPR/Cas9 genome-engineering technology (Maeder and Gersbach, 2016; Jinek et al., 2012). In this manner, our strategy forgoes limitations associated with predicting regulatory features in a genetic locus such as distal enhancers. In addition, this approach abrogates the need to consider limitations on packaging efficiency, as the entire regulatory region need not be packaged in a gene delivery vector. Moreover, by performing targeted integration, this strategy absolves concerns associated with random insertion of provirus within the host genome.

Our use of iPSCs in these studies also provides an important advance, as the base cell population can be precisely defined and potentially undergo additional genome modifications if needed. This approach may prove attractive for regenerative medicine strategies, as clones may be screened for function and then expanded and differentiated toward a variety of terminal cell types to treat multiple tissues from the same engineered cell population. To achieve the same end, prior approaches would require isolation and expansion of primary cells from multiple tissues, followed by treatment of each population with gene delivery vehicles, and finally delivery of engineered cells to the host. This approach lacks the specificity conferred by targeting pre-determined genomic sites for modification using gene-editing nucleases and requires that engineered, primary cells do not senesce prior to serving a therapeutic purpose.

By performing targeted integration to the Ccl2 locus, we rewired the transcriptional circuitry associated with inflammatory signaling in iPSCs. Additional strategies for coupling input/output relationships in cell populations are rapidly emerging in synthetic biology. Continued development of toggle switches (Gardner et al., 2000; Greber et al., 2008; Kobayashi et al., 2004; Kramer et al., 2004), microRNA classifiers (Wroblewska et al., 2015; Xie et al., 2011), and synthetic transcription regulators (Liu et al., 2001; Perez-Pinera et al., 2013a, 2013b; Qi et al., 2013) will facilitate the generation of complex circuits capable of integrating multi-input cues and may enable a cell to discern and respond specifically to not only varying degrees of inflammation, but also a particular type of inflammation. Furthermore, the autoregulated nature of this approach may allow for therapeutic delivery during early, possibly pre-symptomatic stages of diseases, effectively providing the potential for biologic cell-based vaccines for autoimmune diseases. The customization of intrinsic cellular signaling pathways in therapeutic stem cell populations, as demonstrated in this work, opens innovative possibilities for safer and more effective treatments applicable to a wide variety of diseases.

**EXPERIMENTAL PROCEDURES**

**Induced Pluripotent Stem Cell Derivation and Culture** Murine iPS cells were derived and cultured as previously described (Diekman et al., 2012). In brief, tail fibroblasts from adult C57BL/6 mice were transduced with a lentiviral vector driving doxycycline-inducible expression of Oct4 (Pou5f1), Sox2, Klf4, and c-myc (Carey et al., 2009). Pluripotent cells were maintained on mitomycin C-treated MEFS (Millipore) in medium composed of high-glucose DMEM supplemented with L-glutamine, sodium pyruvate, 20% fetal bovine serum (FBS), 100 nM minimum essential medium non-essential amino acids (NEAA; Gibco), 55 μM β-mercaptoethanol (2-ME; Gibco), and 1,000 units of leukemia inhibitory factor (Millipore). A Col2a1-GFP reporter construct (Grant et al., 2000) was transfected into cells by nucleofection, and a clone stably expressing the reporter upon chondrogenic induction was isolated after G418 selection.

**Genome Editing and Clonal Isolation** A plasmid encoding human codon optimized *Streptococcus pyogenes* Cas9 (hCas9) was obtained as a gift from George Church (Mali et al., 2013) (Addgene plasmid #41815). To target hCas9 to the Ccl2 locus, we generated a guide RNA targeting the start codon of the Ccl2 coding sequence using the complementary oligonucleotides sgMcp1-4_S: 5′-caccGCT CTT CCT CCA CCA CCA TGC-3′ and sgMcp1-4_AS: 5′-aaacGCA TGG TGG TGG AGG AAG AGC-3′, where lowercase bases were used to clone into BbsI-generated overhangs in the expression vector (Perez-Pinera et al., 2013a) (Addgene plasmid #47108).

Targeting vectors were produced as described in Supplemental Experimental Procedures.

iPSCs were prepared for transfection by trypsinization followed by a 30-min feeder subtraction. Lipofectamine 2000 (Life Technologies) was used following the manufacturer’s instructions to co-transfect 800 ng of each single guide RNA and 800 ng of hCas9 along with 1.5 μg of the appropriate targeting vector into iPSCs freshly plated on MEFS in complete, antibiotic-free iPSC medium in a 6-well plate. The following day, cells were subjected to selection with 100 μg/mL hygromycin B (Life Technologies), and iPSCs were subcultured on MEFS for 2 weeks prior to clonal isolation.

Clones were isolated either by iterative mechanical picking or by single-cell deposition by a FACSVantage sorter (Becton Dickson). Details for this procedure, as well as clonal screening, are available in Supplemental Experimental Procedures.

**Micromass Pre-differentiation Culture**

Cells were differentiated toward a mesenchymal state using a high-density micromass culture. Cells were cultured in serum-free
a response of engineered cells to IL-1 or TNF-a were subsequently used in monolayer to probe the dynamical

4 ng/mL basic fibroblast growth factor (Roche), and 10% FBS. Cells were subsequently used in monolayer to probe the dynamical response of engineered cells to IL-1 or TNF-a treatment. In addition, cells were used to derive engineered cartilage to evaluate their utility as a source for inflammation-protected tissue regeneration.

NF-kB Activity Assay
A lentiviral NF-kB transcriptional reporter was prepared and transduced into cells as described in Supplemental Experimental Procedures. Luminescence normalized to background levels of no cytokine treatment was used to report induction of NF-kB transcriptional activity.

Chondrogenesis in Aggregate Culture System
Passage 2–3 pre-differentiated cells were trypsinized and resuspended in differentiation medium supplemented with 100 nM dexamethasone and 10 ng/mL TGF-β3 (R&D Systems) at a density of 1 × 10^6 cells/mL. Aggregate cultures were produced by placing cells in wells of a U-bottom 96-well plate (125,000–250,000 cells per well, depending on the experiment) or in 15-mL conical tubes (500,000 cells per tube). Cells were pelleted by centrifugation at 200 × g and cultured for 3–4 weeks prior to treatment with cytokine (0, 0.1–1 ng/mL IL-1α, or 20 ng/mL TNF-α) in the absence of dexamethasone and TGF-β3. Three days later, aggregate cultures and culture supernatant specimens were harvested for gene expression, biochemical, and histological analyses. Detailed methods for these measurements, including ELISA processing, are provided in Supplemental Experimental Procedures.

Statistical Analysis
Statistical analysis was performed with the Statistica 7 software package using ANOVA with Fisher’s protected least significance difference post hoc test with α = 0.05. For qRT-PCR comparisons, fold-change values were log-transformed prior to statistical analysis. Average group values and SEM were calculated in the logarithmic space prior to transforming data to linear values for reporting fold changes.

SUPPLEMENTAL INFORMATION
Supplemental Information includes Supplemental Experimental Procedures and two figures and can be found with this article online at http://dx.doi.org/10.1016/j.stemcr.2017.03.022.

AUTHOR CONTRIBUTIONS

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REFERENCES


Supplemental Information

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Supplemental Figures and Tables

**Figure S1 related to Figure 1:** Ethidium bromide-stained agarose gel demonstrating the result of junction PCR probing for targeted integration of transgenes to the Ccl2 locus. In each reaction, wild-type (WT), Ccl2-Illra, Ccl2-Luc, or Ccl2-sTNFR1 genomic DNA was used as a template. A 2Log Ladder (NEB) was run along with samples and is shown in the right-most lane.

**Supplemental Table Related to Figure 1 and Figure S1:** Primer pairs used in junction PCR to determine the presence of targeted integration events on Ccl2 alleles.

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<th>Target</th>
<th>Forward Primer</th>
<th>Reverse Primer</th>
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<tbody>
<tr>
<td>Illra</td>
<td>5’-TCAGCTGCTGATCTGAGAA-3’</td>
<td>5’-AGGTCAATTAGGACATGTCTA-3’</td>
</tr>
<tr>
<td>Firefly Luciferase</td>
<td>5’-TCAGCTGCTGATCTGAGAA-3’</td>
<td>5’-CAGCGTAAGTQATTCACCTA-3’</td>
</tr>
<tr>
<td>sTNFR1-IgG</td>
<td>5’-TCAGCTGCTGATCTGAGAA-3’</td>
<td>5’-CACTCCTGAGTCCGATC-3’</td>
</tr>
</tbody>
</table>

**Figure S2 related to Figure 3C:** Wild-type cells were treated with a single pulse of cytokine for 24 hours before medium was collected to measure levels of IL-1ra secreted from the endogenous locus of these cells by ELISA. Values presented represent means ± SEM (n = 3).

**Supplemental Table Related to Figures 4 and 5:** Primers used for Gene Expression Assays

<table>
<thead>
<tr>
<th>Target</th>
<th>Forward Primer</th>
<th>Reverse Primer</th>
</tr>
</thead>
<tbody>
<tr>
<td>r18s</td>
<td>5’-CGGCTACCCATCCAGGAA-3’</td>
<td>5’-GGGCTAGAAAGAGTCTCTG-3’</td>
</tr>
<tr>
<td>Acan</td>
<td>5’-GCATGAGAGGCGGATGAG-3’</td>
<td>5’-CTAGATCTCGAGTCACTTTTC-3’</td>
</tr>
<tr>
<td>Adams4</td>
<td>5’-GACCTTCCGTCTAAGCAGTGT-3’</td>
<td>5’-CCTGGCAGTGATTTTCA-3’</td>
</tr>
<tr>
<td>Adams5</td>
<td>5’-GCCCAACCAATGTTAATTCTT-3’</td>
<td>5’-TGACTCCTTTTCATCAGACTGA-3’</td>
</tr>
<tr>
<td>Ccl2</td>
<td>5’-GCCCTCAGGCGATCATGTTAA-3’</td>
<td>5’-CCTACTCATTGAGTACATCTTACTG-3’</td>
</tr>
<tr>
<td>Col2a1</td>
<td>5’-TCCAGATGCACCTCCTCGTCTA-3’</td>
<td>5’-AGGTAGCAGTCTGTTCTTACA-3’</td>
</tr>
<tr>
<td>Illrn</td>
<td>5’-GGCTACCCGAGGTCGTC-3’</td>
<td>5’-TCTCCGAGGTCGTC-3’</td>
</tr>
</tbody>
</table>
Supplemental Experimental Procedures

**Construction of Targeting Vectors.** Targeting vectors were produced in which coding sequences for each transgene (luciferase, sTNFR1-IgG, or IL-1Ra) were cloned directly in place of the start codon of Ccl2. The left homology arm was generated by PCR amplification of the region flanked by the following oligonucleotides from murine genomic DNA isolated using the DNeasy Blood & Tissue Kit (Qiagen): 5'‐AAATTTCTTCTGACCATAGAG-3' and 5'‐CATGTTGGTGAGGAAGAGAGAC-3'. The right homology arm was similarly generated and defined by the following oligonucleotides: 5'‐CAGTTCCCTGTATGCTTG-3' and 5'‐ATCTGGATGATGCTTTGACA-3'. Targeting vectors were produced by isolothermal assembly using pGL3 Basic (Promega) as a backbone. First, PCR fragments including transgenes followed by a Simian virus 40 polyadenylation signal sequence were ligated to the left and right homology arms. Template for the Luciferase transgene was pGL3 Basic. The template for the chimeric human sTNFR1‐murine IgG transgene was a vector provided as a gift from Pascal Bigey (Bloquel et al., 2004). The template for IL-1Ra was cDNA from murine C57Bl/6 mice. The resulting vectors were sequence confirmed, then an expression cassette comprised of the CMV promoter, the hygromycin B phosphotransferase coding sequence, and a bovine growth hormone polyA was cloned into each vector between the left and right homology arms for positive selection of targeted clones.

**Clonal Isolation and Screening.** In preparation for single cell deposition, iPSCs were feeder subtracted prior to culture on 0.1% gelatin for 2 days. Cells were then trypsinized and subjected to a final feeder subtraction and then suspended in calcium- and magnesium-free PBS, 1 mM EDTA, 25 mM HEPES, and 1% FBS. Individual cells were then deposited into MEF-containing wells of a 96-well plate. Clones were sub-cultured on MEFs throughout the screening process. Targeted integration was assayed by performing junction PCR using the oligonucleotides listed in the Supplemental Table Related to figures 4 and 5 (above) for each target. For the PCR, a subset of each clone was lysed using QuickExtract (Epicentre) according to the manufacturer’s instructions. The cell lysate was then diluted 8-10 fold prior to use as template in a PCR using Q5 polymerase (NEB) according to manufacturer’s instruction with the following cycling parameters: 98/30°;98/8°;68/10°;72/20°x35;72/2'. Clones exhibiting unique and specific product from the junction PCR (Figure S1) were propagated on MEFs and until further differentiation. Further analysis of targeting of the Ccl2 alleles in these clones was performed using the following oligonucleotide pair: Surv MCP1 F1: 5'-tcctaggaggtctagaaaa-3'; Surv MCP1 R1: 5'-cctcagcaatctaaaaatgg-3'.

**NF-kB transcriptional reporter construction and use.** A lentiviral construct containing 4 putative NF-kB response elements upstream of firefly luciferase was generated by cloning the following sequence: 5'-CGGGAAAATCCGCCTAGCATGAGGGACTTTTCCCCAATGAGGAAATTAGCCGGGACCTTTCCGTCTCCTGGGAGGGGCCTTCCA-3' upstream of the minimal CMV promoter in pGL3Basic (Promega) and then sub-cloning the cassette including the luciferase transgene into a lentiviral expression vector. Additionally, an NF-kB negative regulatory element (NRE – 5'-AAATCTCTGGA-3')(Noubakhsh et al., 1993) was cloned upstream of the response elements. Lentivirus was generated by co-transfecting 2 μg of the cloned transfer vector, 1.5 μg of psPAX2 (Addgene 12260) and 0.6 μg of pMD2G (Addgene 12259) into 293T cells cultured at confluence in the well of a 6-well plate using Lipofectamine 2000. The next day, medium from 293T virus producer cells was changed, and conditioned medium containing lentivirus was collected approximately 36 and 60 hours after transfection. The lentiviral supernatant was filtered through 0.45 μm cellulose acetate filters and stored at -80°C until use.

Pre-differentiated cells were transduced by supplementing culture medium 1:1 with viral supernatant as well as 4 μg/ml polybrene and incubating the cells in the presence of the virus overnight. Transduced cells were expanded, passaged, and then treated with cytokine. At the indicated time points, samples were lysed and assayed for luminescence using a Bright Glo (Promega) luminescence kit according to manufacturer’s instructions.
**Biochemical analyses of engineered cartilage.** Samples used for biochemical analyses were harvested, rinsed with DPBS, and stored at -20°C until testing. Aggregate culture samples were digested in papain (125 µg/ml; Sigma) at 65°C overnight. Digested samples were then analyzed using the picogreen assay (Lifetechnologies) to measure double-stranded DNA, the ortho-hydroxyproline assay (Woessner, 1961) for measuring total collagen content, and the dimethylmethylene blue assay (Farndale et al., 1986) for measuring the total sulfated glycosaminoglycan content of tissues (n = 3-6 per group).

**Gene expression.** Samples for gene expression analysis were rinsed in DPBS, lysed in cell lysis reagent (Norgen Biotek) and frozen at -80°C until further processing. Total RNA was isolated per manufacturer’s recommendations (Norgen Biotek). Engineered cartilage samples were first homogenized with a pestle. Reverse transcription was performed using the superscript VILO cDNA synthesis kit (Life Technologies) per manufacturer’s instructions. Quantitative RT-PCR was performed with n = 3-4 samples per group on a StepOnePlus using Power Sybr (Applied Biosystems, Inc) per manufacturer’s instructions. Fold changes were determined relative to a reference group cultured without IL-1 and by using 18s rRNA as a reference gene. Gene expression was probed using the primer pairs listed in the Supplemental Table. Primers for the sTNFR1 transgene recognize only the human transgene, and not murine isoforms. Primers for the Il1ra transgene amplify both the Ccl2-driven Il1ra as well as endogenous, murine Il1ra, Il1rn.

**Enzyme-Linked Immunosorbent Assays.** Media samples used in ELISAs were collected from wells and stored at -20°C or -80°C until used. Reagents for ELISAs to detect human sTNFR1 and murine IL-1Ra were purchased from R&D and used according to manufacturer’s recommendations.

**Histological processing.** Samples for histology were rinsed in DPBS upon harvest, fixed in 4% paraformaldehyde for 24 hours, paraffin embedded, and sectioned at 10 µm thickness. Samples were stained with Safranin-O/fast green/hematoxylin using standard protocols.

**Supplemental References**


Woessner, J.F., Jr. (1961). The determination of hydroxyproline in tissue and protein samples containing small proportions of this imino acid. Archives of biochemistry and biophysics 93, 440-447.