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Regulatory T cell-mediated resolution of lung injury: identification of potential target genes via expression profiling

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Acute lung injury (ALI) and acute respiratory distress syndrome (ARDS) manifest as rapid-onset bilateral pulmonary infiltrates and hypoxemia, producing nearly 200,000 hospitalizations and 75,000 deaths in the US each year, with a reported 30–40% mortality (34). ALI is characterized by alveolar-capillary injury, inflammation with neutrophil accumulation, and release of proinflammatory cytokines. Much work has focused on understanding the early, inflammatory phase of ALI, but the resolution phase remains poorly understood. Despite the success of physiological interventions such as low-tidal-volume ventilation in reducing mortality in ALI (1), knowledge of underlying cellular processes defining each phase of ALI, and specifically those required to achieve resolution, is limited. Events specific to repair may be better understood by considering changes in expression of relevant genes (40).

Genomewide measurements of gene expression are powerful tools for assessing global gene changes and have been well characterized in various models of ALI, although largely focused on ventilator-induced lung injury (VILI) (41). Changes in gene expression after intratracheal lipopolysaccharide (IT LPS) have also been described, but most have been limited to the first 24 h after injury (12, 18). To aid in identification of potentially involved genes, one technique involves using gene ontologies (GOs), a method of grouping genes that have common molecular function or participate in similar biological processes, an approach that has been used in selecting process-related candidate genes in VILI (24).

We examined early and late gene expression changes, using the IT LPS model of ALI. In this model, inflammatory injury peaks at day 4 and is almost completely resolved by day 10 in C57BL/6 [wild type (WT)] mice. In contrast to the pattern in WT mice, lymphocyte-deficient recombinase-activating gene-1-deficient (Rag-1−/−) mice exhibit strikingly delayed resolution despite similar initial injury (9). Adoptive transfer of isolated CD4+CD25+Foxp3+ regulatory T cells (Tregs) to Rag-1−/− mice at the time of IT LPS, resolution was similar to that in WT mice. Of the 102 genes distinctly changed in either WT or Rag-1−/− mice from our 7 gene ontologies, 19 genes reverted from the Rag-1−/− to the WT pattern of expression after adoptive transfer of Tregs, implicating those 19 genes in Treg-mediated resolution of ALI.

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Isolation and adoptive transfer of CD4+ CD25+ T cells. For mouse T cells, spleens from CD45.1 mice were removed and prepared for single-cell suspensions. To first isolate CD4+ T cells, CD8 (Ly-2)-, CD11b (Mac-1)-, CD45R (B220)-, CD49b (DX5)-, or Ter-119-positive cells were depleted with biotin-labeled specific MAbs (Miltenyi Biotec, Auburn, CA), anti-biotin magnetic beads, and an LD magnetic bead column (Miltenyi Biotec). To then isolate CD4+CD25+ T cells (Tregs), the purified CD4+ T cell populations were incubated with phycoerythrin (PE)-labeled anti-CD25 antibody (Miltenyi Biotec) and anti-PE magnetic beads and were isolated by MACS separation column (Miltenyi Biotec). The purity of CD4+CD25+ T cell fractions was >95% as assessed by flow cytometry. More than 90% of the CD4+CD25+ cells were also positive for the regulatory T cell transcription factor Foxp3 (9). A single purified cell suspension (1.0 × 10^6 in 100 μl of PBS) was adoptively transferred 60 min after IT LPS via tail vein injection.

Analysis of bronchoalveolar lavage fluid. Bronchoalveolar lavage (BAL) fluid (BALF) was obtained by cannulating the trachea with a 20-gauge catheter after mice were anesthetized and exsanguinated. The right lung was lavaged with two aliquots (0.7 ml) of PBS without calcium; total returns after lavage were 0.8–1.2 ml/mouse. BALF was stored at −80°C. The cell pellet was diluted in PBS, and the total cell number was counted with a hemocytometer after staining with Trypan blue. Differential cell counts were done on cytocentrifuge preparations (Cytospin 3; Shandon Scientific, Runcorn, UK) stained with Diff-Quik stain (Baxter Diagnostics, McGaw Park, IL); 300 cells per sample were counted, and overall percentages were calculated based on three animals per group. Total protein was measured in the cell-free supernatant by the method of Lowry et al. (23).

Lung morphology. Lungs from animals (n = 3–5/sample) at each time point were inflated under a pressure of 25 cmH2O with 1% cell-free supernatant by the method of Lowry et al. (23). The right lung was lavaged with two aliquots (0.7 ml) of PBS without calcium; total returns after lavage were 0.8–1.2 ml/mouse. BALF was centrifuged (700 g, 10 min, 4°C), and cell-free supernatants were stored at −80°C. The cell pellet was diluted in PBS, and the total cell number was counted with a hemocytometer after staining with Trypan blue. Differential cell counts were done on cytocentrifuge preparations (Cytospin 3; Shandon Scientific, Runcorn, UK) stained with Diff-Quik stain (Baxter Diagnostics, McGaw Park, IL); 300 cells per sample were counted, and overall percentages were calculated based on three animals per group. Total protein was measured in the cell-free supernatant by the method of Lowry et al. (23).

Gene chips and hybridization process. Total RNA (0.5 mg) from each sample was labeled with the Illumina TotalPrep RNA Amplification Kit (Ambion) to achieve cDNA synthesis and in vitro transcription. cRNA (single stranded) was generated and labeled by incorporating biotin-16-UTP (Roche Diagnostics). Biotin-labeled cRNA (0.85 mg) was hybridized (16 h) to Illumina’s Sentrix MouseRef-8 Expression BeadChips (Illumina). The hybridized biotinylated cRNA was detected with streptavidin-Cy3 and quantified with Illumina’s BeadStation 500GX Genetic Analysis Systems scanner.

Individual gene array data and gene ontology analysis. Illumina BeadStudio software was used for preliminary analysis. The primary Illumina data returned from the scanner in the form of an “.idat” file, containing a single intensity data value per gene after the computation of a trimmed mean average for each probe represented by a variable number of bead probes per gene on the array. With BeadStudio, information is returned on the number and standard deviation of all the bead measurements per probe per gene, as well as a detection call based on a comparison between the measured intensity calculated for a single probe per gene and the intensities for a large number of negative control beads built into the BeadChip arrays, (D = % above negative/100, I = perfect), and any gene consistently below D = 0.98 for all samples was eliminated from analysis. z-Transformation for normalization was performed on each Illumina sample/array on a stand-alone basis (5). Significantly differentially expressed genes were calculated by satisfying threshold criteria of a z-test P value ≤ 0.001, a false discovery rate (FDR) < 0.1, and either a fold change (FC) ≥ 2.0, ≤ −2.0 or a z-ratio ≥ 3.0, ≤ −3.0. Heat maps were generated with Treeview and Cluster software (15) after row normalization to enhance color pattern.

For generation of GOs, unique IDs for Illumina probes were retrieved with the Mouse database (http://compbio.dfci.harvard.edu). With Microsoft Access, Illumina IDs were converted to Unigene IDs and then assigned to probes comprising the Mouse_Ref-8_V1 microarray. After use of the GenMAPP converting tool (10), MAPP-Finder-compatible files were generated. The software also calculated z-scores based on gene association with one or multiple generated ontologies, and a minimum z-score >1.96 achieved ontology significance (GEO accession no. GSE17355).

Quantitative RT-PCR analysis and correlation to microarray data. Reverse transcription was performed by using total RNA isolated from mouse tissues and processed with the Applied Biosystems (Foster City, CA) High-Capacity cDNA Archive kit first-strand synthesis system for RT-PCR according to the manufacturer’s protocol. Quantitative RT-PCR (qRT-PCR) was performed with the TaqMan assay system from Applied Biosystems. All PCR amplifications were carried out in duplicate on an ABI Prism 7300 Sequence Detection System with a fluorogenic 5’-nuclease assay (TaqMan probes). Probes and primers were designed and synthesized by Applied Biosystems: mu_gapdh (Mm99999915_g1); mu_acb (Mm00438879_s1); mu_pgk1 (Mm00435617_m1); mu_hp (Mm00504051_m1); and mu_fcgr2b (Mm00438656_m1). Relative gene expressions were calculated by using the 2−ΔΔCt method, in which Ct indicates threshold cycle, the fractional cycle number where the fluorescent signal reaches detection threshold (22). The normalized ΔCt value of each sample was calculated by using a total of three endogenous control genes (gapdh, acb, and pgk1). The correlation between microarray and real-time PCR (qPCR) data was analyzed as previously described (24).

Statistical analysis. Differences between groups at each time point were assessed within the same time interval after LPS by the Student’s two-tailed unpaired t-test (SigmaPlot 11.0, Systat software). Figures were generated with SigmaPlot, and data are expressed as means ± SE where applicable. Statistical difference was accepted at P < 0.05. Tables were generated in Microsoft Excel with the sorting function.

RESULTS

Resolution of acute lung injury is impaired in Rag-1−/− mice. Various parameters were quantified to determine differences between WT and Rag-1−/− mice (Fig. 1). After IT LPS, both WT and Rag-1−/− mice lost weight, huddled, and displayed piloerection. WT mice regained weight nearly back to baseline by day 10, with improvement in their appearance, while Rag-1−/− mice exhibited sustained weight loss (Fig. 1A) and continued to appear ill. Lung injury parameters including BAL protein (Fig. 1B) and total cell count (Fig. 1C) were similar between WT and Rag-1−/− mice at day 1, but by day 4 and day 10 only Rag-1−/− mice had persistently elevated protein and total cells. Similarly, day 10 BAL differential cell counts (Fig. 1D) revealed that neutrophil alveolitis, a hallmark of ALL, persisted only in the Rag-1−/− mice despite a significant alveolar neutrophil influx in both groups at earlier time points. Also notable was the increase in BAL lymphocytes seen in WT mice at time points associated with resolution of lung injury; Rag-1−/− mice had no lymphocytes. In summary, lung injury resolution is impaired in lymphocyte-deficient Rag-1−/− mice.
Changes in individual gene expression remain in Rag-1 \(-/-\) mice after acute lung injury. To identify potential differences in gene expression between WT and Rag-1 \(-/-\) mice, we performed microarray analyses of left lung homogenate samples from the same WT and Rag-1 \(-/-\) mice used to generate the phenotypic data shown in Fig. 1. A heat map of 2,000 representative WT and Rag-1 \(-/-\) mice genes revealed predominantly similar gene expression between the mouse strains after IT H2O (Fig. 2A). After IT LPS, differences in gene expression were present as early as day 1 and persisted at day 4. While genes in WT mice returned to baseline expression patterns by day 10, Rag-1 \(-/-\) mouse gene expression remained deviated from baseline.

When we evaluated \(>13,000\) genes on the gene chip for significant injury-mediated gene expression changes versus control gene expression (FC \(\geq 3.3, \leq -3.3\)), we observed substantially more upregulated than downregulated genes in both strains of mice (Fig. 2B). At day 1, more WT genes were significantly changed than Rag-1 \(-/-\) genes, but by day 4 this relationship had reversed. By day 10, significantly changed Rag-1 \(-/-\) mice genes outnumbered significantly changed WT genes by almost 20-fold \((59 \text{ vs. } 3)\). When we displayed the significant genes by Venn diagram, a number of both shared and distinct genes were identified between strains of mice at days 1 and 4 (Fig. 2C). By day 10, however, little gene overlap existed, as only three WT genes were significantly changed.

Gene ontology analysis reveals differences between strains. Given the large number of individual gene expression changes in each group after IT LPS, we extended our analysis to focus on GOs, a grouping of genes with common function or that are present in similar biological processes. ALI causes significant inflammation and damage, leading to a defense response by the immune system. After IT LPS-induced ALI, resolution was markedly delayed in lymphocyte-deficient Rag-1 \(-/-\) mice, perhaps related to an impaired defense response. To identify potential candidate genes that may help explain the impaired response in Rag-1 \(-/-\) mice, we elected to concentrate on seven GOs focused on inflammation or defense/immune responses that were found to be significantly changed based on a GO z-score \(>3.92\) for at least one time point. A listing of all significant gene ontologies for each strain and at each time point can be found in Supplemental Fig. S1.1 The number of significant upregulated and downregulated genes (FC \(\geq 3.3, \leq -3.3\) vs. strain control) is presented for each group and for each time point and for each strain. *\(P < 0.05\) compared with other strain at same time point.

1 The online version of this article contains supplemental material.
We found that analysis of smaller GOs can reveal findings distinct from the larger GO. For example, the innate immune response ontology (Fig. 3E), which is a subset of the immune response ontology (Fig. 3B), had a significant percentage of downregulated genes in the Rag-1\(^{-/-}\) mice at all time points, a finding that was not apparent when assessing the larger immune response ontology. The cellular defense response ontology (Fig. 3F), a subset of the defense response ontology (Fig. 3C), is the only GO listed in which Rag-1\(^{-/-}\) mice had more significantly changed genes at day 1 compared with WT mice. GOs graphed in Fig. 3, A–F, represent general biological processes involved in inflammation and immune response. In comparison, the apoptosis GO (Fig. 3G) represents a more specific biological process potentially important in resolution from lung injury. Yet, despite the specificity, a similar pattern was preserved, as there were more upregulated genes in WT at day 1 but more upregulated genes in Rag-1\(^{-/-}\) at days 4 and 10 (Fig. 3G). In addition to several distinct genes, the apoptosis GO contained genes found in many of the other GOs assessed.

To begin searching for specific genes that may contribute to resolution, we analyzed each of the seven GOs for genes distinctly changed in either WT or Rag-1\(^{-/-}\) mice, but not both, at days 4 and 10 after IT LPS. These 102 significantly changed genes across all seven ontologies are listed by time point in Fig. 4. Supplemental Figure S2 contains lists of changed genes grouped by ontology. When comparing time points after IT LPS, there were nearly three times as many genes distinctly changed in either WT or Rag-1\(^{-/-}\) mice at day 4 versus at day 10. When comparing strains of mice, there were many more genes that were distinctly changed in Rag-1\(^{-/-}\) mice. At day 4, there are more than five times as many significant gene changes in Rag-1\(^{-/-}\) mice. At day 10, there is only one distinctly changed WT gene (Spon2), compared with 25 genes distinctly changed in Rag-1\(^{-/-}\) mice.

Lung homogenate protein changes correlate to gene fold changes after IT LPS. As a method of validation, and to establish significance of gene expression changes, we measured protein levels of three genes identified in the ontology analysis, chosen because of their established roles in inflammatory responses and their presence in multiple GOs in our model. Both FC of gene expression versus control (Fig. 5A) and lung homogenate protein (Fig. 5B) for IL-1\(\beta\), TNF-\(\alpha\), and IFN-\(\gamma\) were measured at the specified time points after IT LPS. Gene expression and protein content for IL-1\(\beta\) were both significantly increased only in the Rag-1\(^{-/-}\) mice at day 10. For TNF-\(\alpha\) and IFN-\(\gamma\), gene expression and protein content were both significantly increased only in the Rag-1\(^{-/-}\) mice at day 4. Overall, significant changes in gene expression correlate with significant differences in protein at specified time points.

Resolution of ALI is achieved after adoptive transfer of Tregs to Rag-1\(^{-/-}\) mice. Rag-1\(^{-/-}\) mice that received adoptive transfer of Tregs on day 0 after IT LPS achieved resolution in a pattern similar to WT mice and significantly improved compared with Rag-1\(^{-/-}\) mice that did not receive Tregs (Fig. 6) or that received other lymphocyte subsets (not shown). Rag-1\(^{-/-}\) mice that received Tregs returned to baseline weight by day 10, similar to WT mice; Rag-1\(^{-/-}\) mice that did not receive Tregs exhibited persistent weight loss (Fig. 6A). In addition, despite similar day 4 BAL protein (Fig. 6B) and day 4 BAL total cell counts (Fig. 6C) in both Rag-1\(^{-/-}\) mice groups, there were significant differences by day 10 consistent

Each of the seven chosen ontologies (Fig. 3). Across ontologies, the number of upregulated genes was greater than the number of downregulated genes. On day 1 after LPS, there were at least as many significantly changed genes in WT mice as in Rag-1\(^{-/-}\) mice in six of seven GOs (except the cellular defense response ontology). In contrast, by day 4, the number of significantly changed genes in each ontology was greater in the Rag-1\(^{-/-}\) mice. Almost all WT genes had returned to baseline by day 10, yet significantly changed Rag-1\(^{-/-}\) genes remained in all seven GOs.
Fig. 3. Changes in gene ontologies persist in Rag-1$^{-/-}$ mice. Gene ontology (GO) analysis combines genes involved in the same biological processes and assesses ontology significance based on overall gene expression changes in that ontology, which is based on a minimum z-score $>1.96$. In each of the 7 ontologies chosen (A–G), significantly up- and downregulated individual genes (FC $\geq 3.3$ or $\leq -3.3$ vs. control) are graphed. In general, each ontology follows the same general pattern; at day 1, there are at least as many significant WT mouse genes as in Rag-1$^{-/-}$ mice. By day 4, this trend has reversed, and at day 10, a number of Rag-1$^{-/-}$ mouse genes remain significantly different from baseline. Very few genes are different from baseline expression in WT mice by day 10. GOs graphed in A–D are larger umbrella GOs, whereas those graphed in E–G are smaller GOs, made up of a subset of genes from the larger GOs.
with resolution only in the Rag-1⁻/⁻ mice that did receive Tregs. Compared with the persistence of BAL neutrophils at day 10 (Fig. 6D) in Rag-1⁻/⁻ mice without Tregs, Rag-1⁻/⁻ mice that received Tregs had decreased BAL neutrophils at day 10, similar to WT mice. Lung histology revealed peak injury that was similar between groups at day 4 and 10. Genes in bold are found in multiple ontologies. Overall, there are almost 3-fold more distinct genes at day 4 (76) compared with day 10 (26). At day 4, there are almost 5-fold more significantly changed genes in Rag-1⁻/⁻ mice compared with WT mice. At day 10, only 1 WT gene (Spon2) is significantly changed; 25 genes remain so in Rag-1⁻/⁻ mice.

**DISCUSSION**

Expression profiling of genes expressed in the lungs in an IT LPS-induced ALI model has been described. Jeysaelen and colleagues (18) as well as Dolinay and colleagues (12) described gene expression in the acute response to LPS. Brass et al. (4) examined patterns of gene expression following inhaled LPS exposure for 1 wk to 2 mo, compared with gene expression following inhalation of bleomycin. Previously, GO analysis has been verified as a useful tool to identify candidate genes in VILI (24). With the use of GOs, we have examined gene expression in the context of resolution of ALI, a poorly understood process of significant clinical importance.

We used a well-established model of IT LPS-induced lung injury (9, 27, 28, 38) with an LPS dose selected to create sufficient injury but with an acceptable mortality to allow study of resolution. We identified a striking delay in resolution of lung injury in lymphocyte-deficient Rag-1⁻/⁻ mice despite similar degrees of initial injury compared with WT mice (9). A prominent feature in this response was the clearance of inflammation from the WT mice, while inflammation persisted in the Rag-1⁻/⁻ mice to day 10. Given the absence of lymphocytes, Rag-1⁻/⁻ mice may have impaired immune responses leading to the marked delay in resolution after ALI. In addition to resolution-phase differences in phenotype after IT LPS, expression profiling of genes in the lung revealed prominent differences between WT and Rag-1⁻/⁻ mice. When we analyzed gene expression to help identify GOs (and resultant biological processes) potentially mediating resolution, we found that both WT and Rag-1⁻/⁻ mice had increased numbers of significantly changed genes in the seven chosen GOs at early time points (days 1 and 4), yet only Rag-1⁻/⁻ mice had persistently activated genes by day 10. At day 10, 25 different genes remained significantly changed compared with baseline expression in Rag-1⁻/⁻ mice, while only 1 remained significant in WT mice. We believe that a return to baseline gene expression seen at day 10 is consistent with the resolution achieved in WT mice; in contrast, a persistent deviation from
baseline expression seen in multiple day 10 genes likely contributes to the impaired resolution in Rag-1−/− mice.

When we adoptively transferred Tregs to Rag-1−/− mice, normal resolution of lung injury was restored. To help identify potential mediators of underlying Treg-dependent resolution, we sought genes that behaved similarly in WT and Rag-1−/− mice that received Tregs and differently from Rag-1−/− mice that did not receive Tregs. Of 102 genes distinctly changed between WT and Rag-1−/− mice at days 4 and 10, 19 behaved similarly in the WT and Rag-1−/− mice that received Tregs.

Given that <20% of genes are similarly changed after adoptive transfer of Tregs to Rag-1−/− mice that received Tregs and that other non-Treg-mediated active resolution processes likely exist, but these were not the focus of our investigation.

Of those 19 potential candidate genes, several interesting and potentially relevant associations were identified that have been described previously. Apolipoprotein E (ApoE) and chemokine ligand 12 (CXCL12) were significantly increased at day 4 only in WT and Rag-1−/− mice that received Tregs but not in Rag-1−/− mice that did not receive Tregs, suggesting a potentially active role in resolution after ALI. ApoE, expressed by type I alveolar epithelial cells, has been shown to be a key mediator in defense against oxidative stress in a rat hyperoxia model (6). Although the involvement of ApoE with Tregs in ALI has not been established, ApoE−/− mice have a reduced number of naturally occurring Tregs at sites of atherosclerosis (29). Given the significant contribution of inflammation in both atherosclerosis and ALI models, it is conceivable that a relationship between Tregs and ApoE expression in the lung exists and helps mediate resolution after ALI. CXCL12, also called stromal cell-derived factor 1 (SDF-1), has been shown to participate in lung repair after bleomycin-induced injury by recruitment of bone marrow-derived stem cells (42). In work by Zou et al. (43), the SDF-1/CXCR4 axis was shown to be

![Graph A](image)

![Graph B](image)
critical in trafficking of Tregs between the bone marrow and the periphery; after IT LPS, CXCL12 may play a role in recruitment of Tregs to the lung and alveolar space, as day 4 gene expression was elevated only in WT mice and Rag-1−/− mice that received Tregs.

In contrast, expression of Adora2b, IL-6, IL8rb, and Nos2 genes was significantly increased at day 4 only in Rag-1−/− mice that did not receive Tregs compared with WT or Rag-1−/− mice that received Tregs, suggesting a potentially active role for these genes in preventing resolution. Adenosine receptor 2b (Adora2b or A2BAR) is protective at early time points in murine VILI and aerosolized LPS models, limiting inflammation and edema formation (14, 35). In contrast, patients with a rapidly progressive form of idiopathic pulmonary fibrosis (IPF) were found to have increased expression of A2BAR on alveolar epithelial cells and fibroblasts compared with those...
with the slowly progressive form of the disease (35). In mice with chronically elevated levels of adenosine, there is upregulation of lung A2BAR and the mice develop rapid and progressive fibrosis (7). IT LPS-exposed Rag-1−/− mice have a more sustained collagen deposition (Movat staining) that persists at day 10 compared with WT mice (9). Upregulation of A2BAR may contribute to the altered collagen regulation in the model. IL-6, although complex in function, has been shown to be associated with increased pulmonary fibrosis, in part mediated by A2BAR expression on lung fibroblasts, suggesting a link between the significant gene expression changes of IL-6 and the mice develop rapid and progressive lung injury in an aerosolized LPS model at 24 h (33). Rag-1−/− mice had increased CXCR2, and neutrophils were reduced after transfer of Tregs. Nitric oxide synthase 2 (Nos2 or iNOS) is the predominant source of nitric oxide (NO) during inflammatory conditions (21). iNOS-null mice have reduced inflammatory responses at early time points of lung injury (16, 19, 21, 31, 39). In contrast, other studies demonstrate a protective role for iNOS in regulation of immune responses to infectious (11, 25) and noninfectious (20) stimuli. Compared with the extensive work on the role of iNOS at early time points, the effects of iNOS on resolution of ALI have not been reported. NO has been shown to induce CD4+CD25+ regulatory T cells from CD4+CD25− T cells in vitro, rendering these cells just as effective in cell suppression as natural Tregs (CD4+CD25+) (30), indicating a potential interaction between Tregs and Nos2.

Expression of Fcgr2b, Fn1, and Ccl3 genes was significantly increased at day 10 only in Rag-1−/− mice that did not receive Tregs, suggesting that these genes may impair resolution after IT LPS. Fc receptor IgG low-affinity II (Fcgr2b, Fcg2r, or CD32) appears to control antibody and cytokine production as well as phagocytosis, and increased mRNA levels are associated with decreased phagocytosis (8). Given that Fcgr2b aids in binding and recruiting neutrophils in cooperation with CXCR2, a potential dual role for this gene in the impaired resolution in Rag-1−/− mice may exist related to both increased recruitment of neutrophils and decreased phagocytosis of neutrophils (17). However, no potential interaction between Fcgr2 and Tregs has been uncovered to date. Fibronectin 1 (Fn1), a high-molecular-weight extracellular matrix glycoprotein, has elevated gene expression

Fig. 7. Based on the 7 GOs and the resulting 102 genes that show differential changes between WT and Rag-1−/− mice from Fig. 4, listed genes show similar expression in Rag-1−/− mice that received Tregs (AT Rag) and WT mice but distinct from Rag-1−/− mice that did not receive Tregs (Rag), for both days 4 and 10. At day 4, 5 genes from WT and Rag-1−/− mice with Tregs are significantly changed (A), and 7 genes from Rag-1−/− mice without Tregs are significantly changed (B). At day 10, 6 genes from WT and Rag-1−/− mice with Tregs genes are significantly changed, and 7 genes from Rag-1−/− mice without Tregs are significantly changed (C). In total, there are 19 potential target genes identified. D: plotted results represent real-time PCR correlation analysis of 24 samples selected from 2 of the 19 gene candidates. Gene expression levels were measured in Fcgr2b and Hp genes. Solid line depicts the position of exact agreement between real-time PCR and Illumina microarray results. Dashed lines indicate the range between 2 SDs (1.96) of differences between transcript detection by microarray and real-time PCR. C, threshold cycle. E: for Fcgr2b, a direct comparison of day 10 gene expression values was made for microarray and RT-PCR techniques for Rag-1−/− mice with and without adoptive transfer of Tregs (E). *P < 0.05 for the labeled comparisons.
in fibrosis-inducing bleomycin and chronic LPS models, suggesting that similar profibrotic mechanisms may exist as part of the impaired resolution seen in Rag-1−/− mice after IT LPS (4). Gene expression of the proinflammatory chemokine (C-C) ligand 3 [Ccl3 or macrophage inflammatory protein-1α (MIP-1α)] was upregulated in a two-hit model using IT LPS followed by mechanical ventilation (2). In conjunction with a study that demonstrated increased lung MIP-1α mRNA levels in the setting of decreased Tregs in a systemic LPS model, the possibility exists that the absence of Tregs in Rag-1−/− mice after IT LPS may directly or indirectly affect the increased MIP-1α observed at day 10 in these animals (32).

It has been suggested that the use of lung homogenates to generate microarrays limits the ability to differentiate whether gene expression changes are derived from resident lung cells or infiltrating inflammatory cells (41). However, our lung preparation involves perfusion with PBS, likely rendering the lung mostly free of nonadherent cells migrating through the vasculature. Although we cannot differentiate genes from adherent inflammatory cells or resident lung cells in the present study, our comparison between two strains of mice with similar baseline gene expression subsequently exposed to the same insult suggests that strain differences in gene expression at later time points serve as one marker of unique biological processes that lead to resolution only in the WT strain. Further evaluation of the candidate genes will be required to identify a cell source. Other studies have utilized microarray analysis of specific cell types in response to LPS in both humans and mice, such as neutrophils (26) and alveolar epithelial cells (13). Although important conclusions can be drawn from such cell-specific studies, we believe that findings in our intact in vivo model provide important insights that will complement subsequent definition of the cellular source of gene expression changes.

In the present study, we have identified differences in individual genes and GOs between WT and Rag-1−/− mice pertinent to the resolution phase of IT LPS-induced ALI in mice. Despite similar initial responses, significant day 4 and day 10 differences in gene expression parallel differences in phenotype between the two strains of mice, with only WT mice having quiescent gene expression and achieving resolution by day 10. Importantly, adoptive transfer of Tregs to Rag-1−/− mice allows the deficient mice to resolve in a manner similar to WT mice (9).

Contrasting these results with findings from Rag-1−/− mice that received transfer of Tregs led to identification of 19 genes that may play a prominent role in Treg-mediated resolution of ALI. As recently described, Tregs appear to orchestrate a complex series of events to mediate resolution of ALI (9). These studies provide new insights into potential mechanisms responsible for that clinically important problem.

GRANTS

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DISCLOSURES

No conflicts of interest are declared by the author(s).

REFERENCES


