Anti-inflammatory therapies of amyotrophic lateral sclerosis guided by immune pathways

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Abstract: Sporadic ALS patients display heterogeneous immune pathways in peripheral blood mononuclear cells (PBMCs). We tested nine sALS patients and one unaffected identical twin of an index case by RNA-Seq of PBMCs. The inflammatory patients (n = 3) clustered into a subset with an inflammatory Th1/Th17 signature and the non-inflammatory patients (n = 7) into another subset with a B cell signature. The inflammatory subset was remarkable for granulocyte and agranulocyte diapedesis, hepatic fibrosis, roles of cytokines and metalloproteases. The non-inflammatory subset was highlighted by degradation of vitamin E, serotonin and nucleotides, altered T cell and B cell signaling, agranulocyte diapedesis, and up regulation of B cell genes. Identification of these differentially regulated pathways in sALS patients may guide the choice of anti-inflammatory therapies.

Keywords: Amyotrophic lateral sclerosis, immune pathways, tocilizumab, hepatic fibrosis, vitamin E

Introduction

The presence of inflammation in the spinal cord of sporadic amyotrophic lateral sclerosis (sALS) patients is documented by immunohistochemical demonstration of activated microglia, astrocytes, and activated complement components [1], along with the infiltration by dendritic cells, macrophages [2], and IL-17A-positive CD4 and CD8 T cells and mast cells [3]. Inflammatory macrophages phagocytize both normal and apoptotic neurons in the ALS spinal cord [4] and infiltrate the spinal cord of sALS patients [3] and animal models of ALS, contributing to disease progression [5, 6]. Others claim that only T cells cross the blood-brain barrier and activate microglia [7], but this is inconsistent with the results in experimental models [5, 6] and infiltration by monocyte/macrophages in the ALS spinal cord [8]. The inflammatory cytokines IL-1 and IL-6 are induced in peripheral blood mononuclear cells (PBMCs) of sALS patients by mutant or aggregated wild-type superoxide dismutase-1 (SOD-1) [3]. IL-6 and TGF-β are present in the serum of sALS patients early in the disease and IL-17A is found in the mid-course of the disease [4]. Th17 cells have been increasingly found in ALS patients [3, 9, 10], and ALS has been associated with prior history of autoimmune diseases, including multiple sclerosis, myasthenia gravis and systemic lupus erythematosus [11].

Thus inflammation, including autoimmune inflammation, is documented in sALS but anti-inflammatory therapy has not yet been proven successful. A recent study identified heterogeneous, Th1, Th17, and IL-6 driven inflammatory pathways in ALS patients [10]. Specific neurotoxic mechanisms include Th17 cell disruption of the blood-brain barrier [12] and IL-6 trans-signaling [13]. Although the clinical trial of celecoxib failed [14], recent anti-inflammatory approaches against ALS are promising [15, 16]. In addition, the lipidic mediator resolvin D1 (RvD1) attenuated IL-6 and TNF-α production in ALS macrophages, suggesting beneficial role of omega-3 supplementation [4].
The recognition of IL-6-driven inflammation in inflammatory sALS patients [16] has stimulated a pending clinical trial of the IL-6 receptor antibody tocilizumab (Actemra®). As shown in a previous cross-sectional study, one subgroup of “inflammatory” sALS patients had a highly increased expression of inflammatory cytokines, in particular IL1, IL6, and IL8, chemokines, metalloproteinases and transcription factors, whereas the “non-inflammatory” group had a near normal expression, except for increased chemokines CXCL9, CXCL10 and CXCL11 [17]. Two “inflammatory” patients responded to Actemra® therapy by down regulation of inflammatory genes [16]. To develop a scheme for sub grouping sALS patients for an appropriate anti-inflammatory therapy, we have investigated the transcriptome of ALS patients’ PBMCs for the signatures of signaling pathways.

Materials and methods

Patients and controls

The immune and genetic investigation had institutional and ethical review board approval. The study population included ALS patients from a previous study [16] and a pair of twins discordant in the diagnosis of ALS (Figure 1A). The assignment to the previously described inflammatory group was based on RT PCR expression of IL-1β and IL-6 in PBMCs [16] with the threshold value (Ct) at most time-points < 24 cycles; in “non-inflammatory” patients, the threshold Ct was ≥ 24 cycles. Three patients received Actemra® infusions from their private physicians.

RT PCR assay of inflammatory gene mRNAs

The assay was done using a custom array of inflammatory genes (SABiosciences) on the Roche LightCycler using the ΔΔCt method [29]. A lower number of cycles (Ct) indicate a higher inflammation.

Th17 cell assay

PBMCs were isolated by the ficoll-hypaque gradient method, washed with 1XPBS and re-suspended in completed medium. 0.5-1.0×10⁶ cells were incubated with medium, or medium with superoxide dismutase1 (SOD-1) or SOD-1 (2 µg/ml) plus tocilizumab (10 µg/ml) for 20 hours, and in presence of Brefeldin A (Golgi-plug) (1 µl/ml) (BD Biosciences) for last 6 hours. The cells were harvested and washed with a FACS buffer (PBS in 0.02% NaN₃ (wt/vol) and 0.5% BSA), labeled with FITC-conjugated anti-CD3 antibodies, washed and permeabilized with cytofix/cytoperm (BD Biosciences) solution according to the manufacturer protocol and stained by PE or APC-conjugated anti-IL17 (BD Biosciences, San Diego, CA). Flow cytometry was performed using a FACSCalibur instrument. Data were analyzed using FlowJo software (Ashland, OR) with lymphocyte gate, based on forward and side scatter.

Patient expression profiling

RNA-seq was performed on patient PBMCs using standard Illumina RNA-seq library construction protocols. RNA-seq libraries were sequenced on illumina HiSeq 2000. Reads were aligned to the hg19 reference genome using TopHat [30]. Gene counts were quantified with HTSeq and normalized with DESeq, followed by adding a pseudo count of 1 for each gene [31]. To cluster the patient PBMC RNA-Seq data, gene expression signatures were identified from microarray intensity values of published works in which PBMC derived monocytes were stimulated by IL4 (IL4_06, IL4_24), IL10 (IL10_06, IL10_24), IL15 (IL15_06, IL15_24), NOD2L (NOD2L_06, NOD2L_24), TLR2/1L (TLR2/1L_06, TLR2/1L_24), Vitamin A (VIT_A), or Vitamin D (VIT_D) at different periods of exposure [18-20]. fRMA was used for normalization of the microarray intensity values [32]. To identify genes highly expressed in the stimulated monocytes, the ratio of the median intensity value of a perturbation set to the median intensity value of their respective control set was used to rank the genes. The top 50 ranking genes were chosen for each perturbation to serve as the signature for high expression. Additional signatures include the genes induced by interferon-β (IFN-β) and interferon-γ (IFN-γ) from IFN-treated PBMCs [33]. For each patient, a signature expression value was determined by calculating the arithmetic mean of the log₁₀ gene count across the 50 genes within a signature for each patient. Patients were subsequently clustered on their set of mean signature values distanced by correlation.

Pathway enrichment analysis

Differentially expressed genes between the inflammatory patients (1, 5, and 11) and non-
Immune pathways in ALS

**Figure 1.** Unsupervised clustering of patient population. A. Patient disease status and demographics ordered by correlation-based cluster of PBMC gene counts. B. Hierarchical clustering dendrogram of ALS blood expression measured by their relative enrichment (Z-score) for immune perturbation signatures across the ALS samples. Unique gene signatures derived from adherent PBMC stimulated by perturbation at 6 h or 24 h as indicated. Each square represents the enrichment score of the top 50 unique genes of a signature (column) for a patient (row) with respect to the other patients, where red indicates a higher enrichment and blue indicates a lower enrichment score of the signature genes. Patients are organized into two main clusters by correlation, where magenta includes inflammatory patients 1, 5 and 11. The orange cluster includes non-inflammatory patients 6, 12, 13, 14, 15, 16 and 18. C. Transcriptional responses of the downstream genes in the IL-15 pathway. Note downregulation of IL-6 responses but heterogeneity in IL-1beta responses in “non-inflammatory” patients.

Inflammatory patients (6, 12, 13, 14, 15, 16, and 18) were detected using DESeq (Anders et al. 2010). The genes were ranked by the fold change expression of the inflammatory patients to the non-inflammatory patients. To identify pathways enriched in the high expression genes among the inflammatory patients, the top 750 genes with a log2 fold change > 2 and an FDR < 0.05 were submitted to QIAGEN’s Ingenuity® Pathway Analysis (IPA®, QIAGEN Redwood City, www.qiagen.com/ingenuity). The bottom 539 genes with a log2 fold change < -2 and an FDR < 0.05 were submitted to Ingenuity’s pathway analysis tool to identify en-
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riched pathways for higher expression genes among the non-inflammatory patients.

Results

Clustering of sporadic ALS patients according to the signatures of Th1 and B-cell pathways

Clinical and demographic data of nine sALS patients and one identical non-affected twin of the patient #5 from a previous study [16] are displayed (Figure 1A). Through an unsupervised clustering method, the sALS patients #6, 12, 13, 14, 15, 16 and 18 were tightly clustered together based on the expression of 25,367 genes, while sALS patients #1, 5, and 11 formed a second cluster (Supplemental Figure 1).

To characterize the inflammatory state of the sALS patients, we intersected their PBMC mRNA expression profiles with gene expression signatures induced by activation of immune cells. These signatures are derived from previous studies involving the stimulation of adherent PBMCs with Th1- and Th2-like cytokines (IL-4, IL-10, IL-15, IFN-β, or IFN-γ), microbial ligands to innate immune receptors (NOD2L, TLR2/1L), and the vitamins A and D [18-20]. We generated a list of unique signature genes that were upregulated in response to each of these perturbations (see Methods). The relative enrichment score (Z-score) of each perturbation signature across the ALS blood samples was clustered and represented as a heat map of the enrichment score (Figure 1B), in which the ALS profiles are clustered based on their signature enrichment. Strikingly, we find that this analysis also generated two contrasting groups, much as the previous one in Figure 1A. This allows us to characterize the larger of the two groups (patients 16, 13, 18, 6, 14, 12, and 15) as B-cell “noninflammatory”, as it has lower levels of IL-15, NOD2L, and TLR2/1L-induced gene signatures in comparison to the Th1 (patients 5, 11, and 1) “inflammatory” group (Figure 1B). We find that the cytokine response signatures of the noninflammatory group are more heterogeneous. The patient #6 had elevated levels of IL-4 and IFNγ signatures, the patient #15 showed higher levels of the vitamin A program, and the patients #6, 12, 16, and 18 had higher levels of both the ligands APRIL and BAFF and the receptors BCMA and TACI (Supplemental Figure 2). The transcriptional responses of the down-stream genes in the IL-15 pathway (Figure 1C) separate the patients in a greater detail and confirmed that some patients had a mixed pattern (e.g. patient #6 and #15).

Clustering of sporadic ALS patients with follow-up samples

A follow-up set of RNA-Seq samples were available for patients #18 and 14 and clustered on the expression of 25,367 genes along with other patient samples. The paired set of samples 18_R1, 18_R2, and 14_R1, 14_R2 reveal tight clustering within patients that is indicative of high correlation in gene expression profiles from the same individual at different time points (Supplemental Figure 1). This suggests that the gene expression signatures are quite robust over time, although they manifest some differences resulting from the treatment or disease progression.

Enriched pathways of high expression genes in inflammatory patients

We next asked whether the genes that were differentially expressed in the inflammatory and noninflammatory groups were enriched for certain functions and pathways using the Ingenuity Pathway Analysis tool (QIAGEN’s Ingenuity Pathway Analysis IPA, QIAGEN Redwood City <http://www.qiagen.com/ingenuity>. Accessed 2015 Mar 5). We found that the pathways enriched among the inflammatory patients include granulocyte and agranulocyte adhesion and diapedesis, hepatic fibrosis, differential regulation of cytokine production in macrophages and T helper cells by IL-17A and IL-17F, inhibition of matrix metalloproteases, atherosclerosis signaling, and IL6-signaling (Figure 2A). Genes among the IL-6 signaling pathway include IL6 itself and many genes downstream of the IL-6 responsive element (TSG6, COL1A1, and IL8) (Figure 2B). The differential regulation of cytokine production in macrophages and T helper cells by IL-17A and IL-17F pathways include pro-inflammatory genes that are induced in macrophages by either IL-17A or IL-17F (IL-1β, IL-6, IL-10, IL-12, IL-13, CCL2, CCL3, and G-CSF) (Figure 2C).

Enriched pathways of high expression genes in non-inflammatory patients

Pathways enriched among the genes that have higher expression in non-inflammatory patients
Immune pathways in ALS

A

- Granulocyte Adhesion and Diapedesis
- Agranulocyte Adhesion and Diapedesis
- Diff. Reg. of Cytokine Prod. in Macrophages and T Helper Cells (IL-17A, IL-17F)
- Hepatic Fibrosis / Hepatic Stellate Cell Activation
- Role of Cytokines in Mediating Communication between Immune Cells
- Differential Regulation of Cytokine Production in Intestinal Epithelial Cells by IL-17A
- Atherosclerosis Signaling
- LXR/RXR Activation
- Inhibition of Matrix Metalloproteinases
- Role of Hypercytokinemia/hyperchemokinemias in the Pathogenesis of Influenza
- HMGBl Signaling
- Bladder Cancer Signaling
- HIF-1 Signaling
- Role of IL-17A in Arthritis
- Role of IL-17F in Allergic Inflammatory Airway Diseases
- Hepatic Cholestasis
- Communication between Innate and Adaptive Immune Cells
- Role of IL-17A in Psoriasis
- Acute Phase Response Signaling
- Role of Osteoblasts, Osteoclasts and Chondrocytes in Rheumatoid Arthritis
- Role of Macrophages, Fibroblasts and Endothelial Cells in Rheumatoid Arthritis
- IL-4 Signaling
- Dendritic Cell Maturation
- IL-8 Signaling

B

IL-6 Signaling

C

Diff. Reg. of Cytokine Prod. in Macrophages and T Helper Cells (IL-17A, IL-17F)
Immune pathways in ALS

**Figure 2.** Pathway Enrichment of Higher Expression Genes in Inflammatory Patients. A. The top 25 significant canonical pathways from the high expression genes in inflammatory patients. B. IL-6 signaling pathway. Highly expressed genes in inflammatory patients are marked in purple. Omitted from the figure is the pathway involved in T helper cells. C. Differential regulation of cytokine production in macrophages and T helper cells by IL-17A and IL-17F pathways.

include alpha-tocopherol degradation, altered cytokine signaling, agranulocyte adhesion, serotonin degradation, retinoate biosynthesis, B cell development, atherosclerosis signaling, and other pathways (Figure 3A). Highly expressed genes in non-inflammatory patients include (a) the genes CYP4F2 and CYP4F3 involved in alpha-tocopherol degradation (Figure 3B); (b) the genes in the B cell development pathway including the members of MHCII (HLA-DMA, HLA-DMB, HLA-DRB1, HLA-DRB5) (Figure 3C); and (c) the genes SULT1A1, SULT1B1, DHR59, ALDH2, and ALDH1A1 involved in serotonin degradation (Figure 3D). In addition, the ligand for the B cell developmental pathway (APRIL) and the receptors (BCMA/TNFRSF17 and BCR) were heterogeneously overexpressed in non-inflammatory patients (Supplemental Figure 2).

Th17 cells are increased in the inflammatory subgroup and are inhibited by tocilizumab

In freshly isolated PBMCs, Th17 cells were increased in the inflammatory subgroup patients in comparison to the non-inflammatory group patients and control subjects (Figure 4). After overnight stimulation by fibrillar SOD-1, Th17 increased 2- to 5-fold in the inflammatory subgroup subjects but did not change significantly in the non-inflammatory subgroup and control subjects. Overnight treatment with tocilizumab reduced (N.S.) Th17 induction by SOD-1 in the inflammatory subgroup, but did not have an effect in the non-inflammatory subgroup.

Inflammatory cytokines are down-regulated by Actemra® therapy in the “inflammatory” subset but not in the “non-inflammatory” subset

A brief follow-up of patients #1 and #6 was previously published [16]. Here we show that on a longitudinal follow-up of the inflammatory patient #1, and the non-inflammatory patients #6 and 14, inflammatory or non-inflammatory transcription, respectively, appeared to be a stable biomarker of these sALS patients in the course of the disease (600-1400, 100 to 600, and 750 to 950 days after onset, respectively) (Figure 5). Extended follow-up of the inflammatory patient 1 revealed up regulation of inflammatory cytokines on entry into the Actemra® therapy in May 2012 (913 days after onset), but, following the start of therapy, down regulation of inflammatory cytokines for 4 months with immediate down regulation 2 hours after infusion of Actemra (see Figure 5D). The patient then developed a brief resistance to Actemra® therapy for 2 months followed again by attenuation of inflammation. However, he stopped therapy in February 2013 (1200 days after onset) and afterwards became more inflammatory and expired 1930 days after onset. Two non-inflammatory sALS patients were given a short course of Actemra® therapy. They had low transcription of inflammatory cytokines on entry into the therapy, but their inflammatory cytokine transcription actually increased during the therapy, which was then stopped (Figure 5B and 5C). To determine the immediate response to Actemra, inflammatory cytokine transcription was measured before and 1-2 hr after treatment. For patient 1, the IL1A and IL8 transcription decreased immediately after the infusion (Figure 5D). For patient 6, all inflammatory cytokine, with the exception of IL1A, increased following the infusion (Figure 5E).

Discussion

In this study, we confirmed the heterogeneity of inflammation in sALS patients, which was previously observed and believed to be important for selection of appropriate patients for tocilizumab (Actemra) therapy [16]. Only “inflammatory” patients are likely to respond to Actemra. Although inflammation has been demonstrated in a longitudinal study [16] and cross-sectional studies of sALS patients [10, 21], the progression of inflammation since the beginning to the end has not been completely analyzed. Here we observe that the inflammatory or non-inflammatory phenotype was maintained throughout the observed disease course, which however, started only 600 days after onset in the “inflammatory” patient. Thus, it is still plausible that a non-inflammatory phase preceded the inflammatory phase early after onset, as described by S. Appel's group [22]. It will be important to fol-
Figure 3. Pathway Enrichment of Higher Expression Genes in Non-Inflammatory Patients. A. The top 25 significant canonical pathways from the high expression genes in non-inflammatory patients. B. α-tocopherol (vitamin E) degradation. C. B-Cell development pathway with high expression genes in the non-inflammatory patients marked in purple. D. Serotonin degradation pathway. Highly expressed genes in inflammatory patients are marked in purple. E. Retinoate Biosynthesis I pathway. Highly expressed genes in inflammatory patients are marked in purple.

low ALS patients over time for establishing their “inflammatory” phenotype, as “non-inflammatory” ALS patients may become “inflammatory” and respond to Actemra therapy.

The “inflammatory” patient #1 had inflammatory phenotype before and after treatment with Actemra®, but became less inflammatory during Actemra® therapy, except for a transitory
resistance to this therapy probably due to reduced uptake and degradation of IL-6 caused by tocilizumab [23]. The “inflammatory” patients had more Th17 cells in comparison to “non-inflammatory” patients. Tocilizumab therapy in vitro and in vivo attenuated Th17 cell induction as well as inflammatory cytokines. The patients in the “non-inflammatory” subgroup had low transcription and production of inflammatory cytokines and chemokines at baseline but, in response to the Actemra® therapy, two patients actually increased the transcription of IL-6 and other inflammatory cytokines.

The up regulated Th1 and B-cell pathways clarify certain features of the pathophysiology of sALS patients. In both groups of patients, immune cell adhesion and diapedesis explain the infiltration by immune cells (granulocytes and agranulocytes) of the affected spinal cord segments [3]. In the inflammatory group, hepatic fibrosis and stellate cell activation is consistent with fibrosis and liver dysfunction in sALS patients [24]. Atherosclerosis signaling could be related to vascular changes with downregulation of tight junctions caused by SOD-1 in an animal model [25] and to inflammation caused by SOD-1 in human macrophages in vitro [3]. Increased α-tocopherol degradation may lead to deficiency of vitamin E. Patients with familial isolated vitamin E deficiency have neurological disorders characteristic of vitamin E deficiency such as cerebellar ataxia and dysarthria [26]. Importantly, in a large study of ALS incidence, long-term vitamin E supplement use was associated with lower ALS rates [27]. Increased serotonin degradation may be related to depression, which is common in ALS patients, as 37% patients are taking anti-depressant medication [28]. Increased retinoate may contribute to osteoporosis in ALS patients.

In conclusion, this study describes heterogeneity of signature pathways in sALS patients that may inform personalized therapy with drugs targeting various pathways, including inflammatory, vitamin, cytochrome P450 superfamily of enzymes, and B-cell developmental pathways. Currently, tocilizumab (Actemra) has shown positive effects in Th1 patients. However, tocilizumab would not be beneficial in the non-inflammatory subset of patients who might benefit from other approaches, such as a therapy targeted at B cells, since the B cell activation and survival genes were upregulated in these patients. These results provide a new rationale for a targeted molecular therapy of inflammatory sALS patients guided in a personalized fashion by the PBMC transcriptome.

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Disclosure of conflict of interest

M.F. received paid travel to meetings and honoraria for consultations from Smartfish AS, Oslo, Norway.

Authors’ contribution

MF and MP designed research. LL and DM performed bioinformatics analysis. RH, LR, AR per-
formed molecular studies. MF, MP, LL, BS, SW, and RR analyzed the data. MF, MP, LL, RRS wrote the paper. All authors read and approved the final manuscript.

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Supplemental Figure 1. Clustering of patient population with follow-up samples. Correlation based cluster of PBMC gene counts of UCSC hg19 genes with the patient initial RNA-Seq samples along with follow-up results for (A) patient 14 (14_R2) and (B) patient 18 (18_R2).
Supplemental Figure 2. Transcriptional responses of Th1 and Th2 genes in individual ALS patients. Differential responses by the cytokines, the chemokine CCL20, the enzyme CYP4F2, and the genes in B cells developmental pathway. A. Transcriptional response high in the inflammatory patients (IL1A, IL1B, IL6, CCL2). B. Transcriptional responses high in the non-inflammatory patients (CYP4F2, APRIL, BCMA, BCR).