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cFLIP expression is altered in severe corticosteroid-resistant asthma

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ABSTRACT

Dysregulation of alternative splicing of mRNA precursors is known to contribute to numerous human diseases. In this study we carried out the first systematic search for asthma-associated changes in alternative splicing events, using a model of *Aspergillus fumigatus* (*A. fumigatus*)-sensitized mice and an exon junction microarray to detect potential changes in alternative splicing. One of the sensitization-associated changes identified in the search was a shift in alternative splicing of the mRNA encoding cFLIP, a modulator of the caspase-mediated extrinsic apoptosis pathway. Expanding these studies to human asthma patients, we discovered a significant decrease in the expression of both cFLIP isoforms in severe corticosteroid-resistant asthmatics. Although it is unclear whether these changes were due solely to differences in alternative splicing, these findings provide evidence that dysregulation of the extrinsic apoptosis pathway is part of the underlying immunopathogenesis of severe refractory asthma.

Introduction

Investigations of the genetic processes that underlie various aspects of asthma have concentrated mainly on the identification of disease-associated genes. These studies have involved either linkage analysis (e.g., [35]), or, on a larger scale, microarray-based gene expression screens (e.g., [3]). Typically the expression levels of identified genes were monitored subsequently through the course of the disease or correlated with its severity (e.g. [2,13]). However, little attention has been given to specific avenues of gene control, notably including possible changes in alternative splicing of mRNA precursors.

Alternative splicing is the major contributor to vertebrate protein diversity (reviewed in [21,27]). According to recent estimates, at least 95% of human pre-mRNAs containing multiple exons are subjected to alternative splicing resulting in multiple mRNA isoforms [29]. Inclusion, omission or exchange of functional domains through alternative splicing has been shown to affect every facet of protein function including enzymatic activity [28], subcellular localization [32], ligand affinity [22] and specificity [23], and many others.

Alternative splicing defects have been linked to multiple genetic disorders. These include ß-thalassemia, spinal muscular atrophy and type 1 neurofibromatosis, among others (reviewed in [4,40,43]). Transcripts encoded by several asthma-associated genes also undergo alternative splicing [20,31,34,36,37,39,44], although only in the case of cysteinyl leukotriene type I receptor does there seem to be a connection to asthma immunopathogenesis [34]. These data, coupled with the enormous biological and pathological importance of alternative splicing, suggest that it is highly likely that alternative splicing contributes to asthma pathogenesis.

Many genetic disorders appear to result from dysregulation of a single transcription or alternative splicing event and, consequently, a relatively straightforward disease pathway. In contrast, asthma is increasingly thought to stem from a complex interplay of multiple genetic events, some of which can be very subtle. In the absence of a single well-defined disease pathway, the identification of underlying alternative splicing events requires both a well-defined pathological model and a comprehensive approach that would allow parallel examination of multiple events. The potentially very large number of mRNA isoforms originating from a single gene, the levels of which have to be measured independently, further complicates analysis of alternative splicing. In addition, changes in isoform ratio, that are indicative of changes in alternative splicing, have to be discerned from changes in the overall transcription or mRNA stability from a single gene. Finally, the levels of minor mRNA isoforms can be very low, requiring an amplification step to ensure detection.

In order to minimize all of the above problems we chose to use an exon junction microarray-based screening system, originally developed for identification of cancer-related alternative splicing events [9]. These microarrays are designed to independently test the levels of sequences

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that uniquely form during resolution of each alternative splicing event; specifically, the junction of two exons, at least one of which can be alternatively included or omitted from the final mRNA. This technology allows unbiased assessment of levels of each of the two isoforms that may result from a particular alternative splicing event. In addition, the design of the microarray provides for PCR amplification of target sequences, thus facilitating detection of signals from low abundance of RNA isoforms.

We now have carried out the first systematic search for asthma-related changes in alternative splicing. Using the above-described microarray strategy together with a common animal model of asthma, based on *Aspergillus fumigatus* (*A. fumigatus*)-sensitized mice [17], we identified a sensitization-dependent change in alternative splicing of the mRNA encoding cFLIP, a modulator of the extrinsic apoptosis signaling pathway. Extending these results we observed an asthma-dependent decrease in overall levels of both cFLIP mRNA isoforms in human severe corticosteroid resistant asthmatics. Our findings provide novel evidence that dysregulation of the cFLIP expression pathway, possibly reflecting changes in alternative splicing, may contribute to development of asthma pathology.

Results and discussion

We first set out to identify gene transcripts that might display asthma-related changes in alternative splicing. Our initial approach was to use splenic samples from A. *fumigatus* sensitized mice [17,19], together with a mouse exon junction microarray annotated without bias [9]. Mice were sensitized through nasal administration of *A. fumigatus*, as described in Materials and Methods, and allergic sensitization was monitored through measurement of serum immunoglobulin (Ig) E levels (Fig. 1). Subsequently, total RNA was collected from the spleen of nine control and seven sensitized mice and the samples were applied to mouse exon junction microarray capable of independently testing levels of 3000 individual mRNAs produced from nearly 1000 genes. The microarray screen displayed statistically significant *A. fumigatus*-dependent changes in levels of many individual mRNAs (e.g., 48 with $p < 0.0001$, data not shown). Further analysis (see Materials and Methods) identified 16 genes that displayed statistically significant shifts in mRNA isoform ratios between control and sensitized groups. Thirteen of these genes that have homologous alternative splicing events in humans were selected for further verification.

Verification of the microarray results was carried out through quantitative RT-PCR assays using an expanded panel of mice samples (11 control, 12 sensitized) that also included all the samples originally used with the microarray. For various technical reasons (e.g., very low levels of one or both isoforms), we were able to test successfully only six sets of isoforms by this method (Supplementary Table 2). Ultimately, we confirmed *A. fumigatus*-dependent changes in alternative splicing of transcripts from two of the 13 tested genes: Eukaryotic translation initiation factor 4A (eIF4A) and, with moderately higher confidence, cellular FLICE-like inhibitory protein (cFLIP), also known as CFLAR (CASP8 and FADD-like apoptosis regulator). For reasons described below, we pursued analysis of the cFLIP gene and its possible link to asthma.

The cFLIP alternative splicing event involves either removal of intron 5 (and splicing of exon 5 to exon 6), yielding $cFLIP_{Long}$ mRNA, or retention of a segment of this intron immediately downstream of constitutive exon 5 with subsequent utilization of a cleavage/polyadenylation site positioned in the intron, forming $cFLIP_{Short}$ mRNA (Fig. 2A). Microarray measurements indicated the levels of both cFLIP isoforms decreased after *A. fumigatus* sensitization; however, the $cFLIP_{Short}$ mRNA level decreased nearly 3-fold, while that of $cFLIP_{Long}$ decreased less than two-fold, resulting in a significant change in the ratio of the two isoforms (Fig. 2B). Quantitative RT-PCR experiments showed no statistically significant change in $cFLIP_{Long}$ mRNA levels, while $cFLIP_{Short}$ mRNA displayed a statistically significant ($p = 0.0019$) 20% decrease in sensitized mice (Fig. 2C).

While the relative decrease of $cFLIP_{Short}$ isoform was observed by both microarray and RT-PCR, only the former assay displayed a concomitant decline of $cFLIP_{Long}$ mRNA. We consider the quantitative RT-PCR results to be a more accurate measurement of changes in mRNA levels because a greater number of samples were tested by this method. Furthermore, unlike the microarray, this assay tested a particular target and thus is less likely to generate artifacts. We thus conclude that *A. fumigatus* sensitization leads to a shift in relative levels of cFLIP mRNA isoforms, most likely affecting alternative splicing of cFLIP and resulting in higher relative levels of $cFLIP_{Long}$.

The two cFLIP isoforms also are produced in humans, but through a different alternative splicing pathway. In this case the short isoform is formed by inclusion of $cFLIP_{Short}$-specific exon 5A followed by a cleavage/polyadenylation site. In $cFLIP_{Long}$, exon 5A and surrounding sequences are skipped entirely through splicing of constitutive exon 4 to exon 5B (Fig. 3A). To examine whether alternative splicing of human

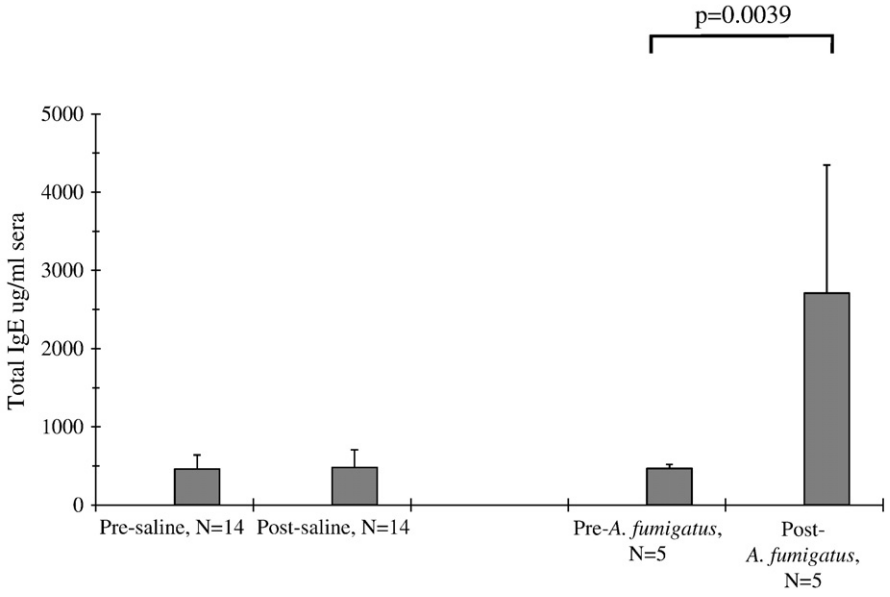

Fig. 1. Development of airway inflammation in mice through *A. fumigatus* sensitization. Changes of serum IgE levels were monitored in mice before and after nasal administration of either PBS or *A. fumigatus* in six doses at 4-day intervals.

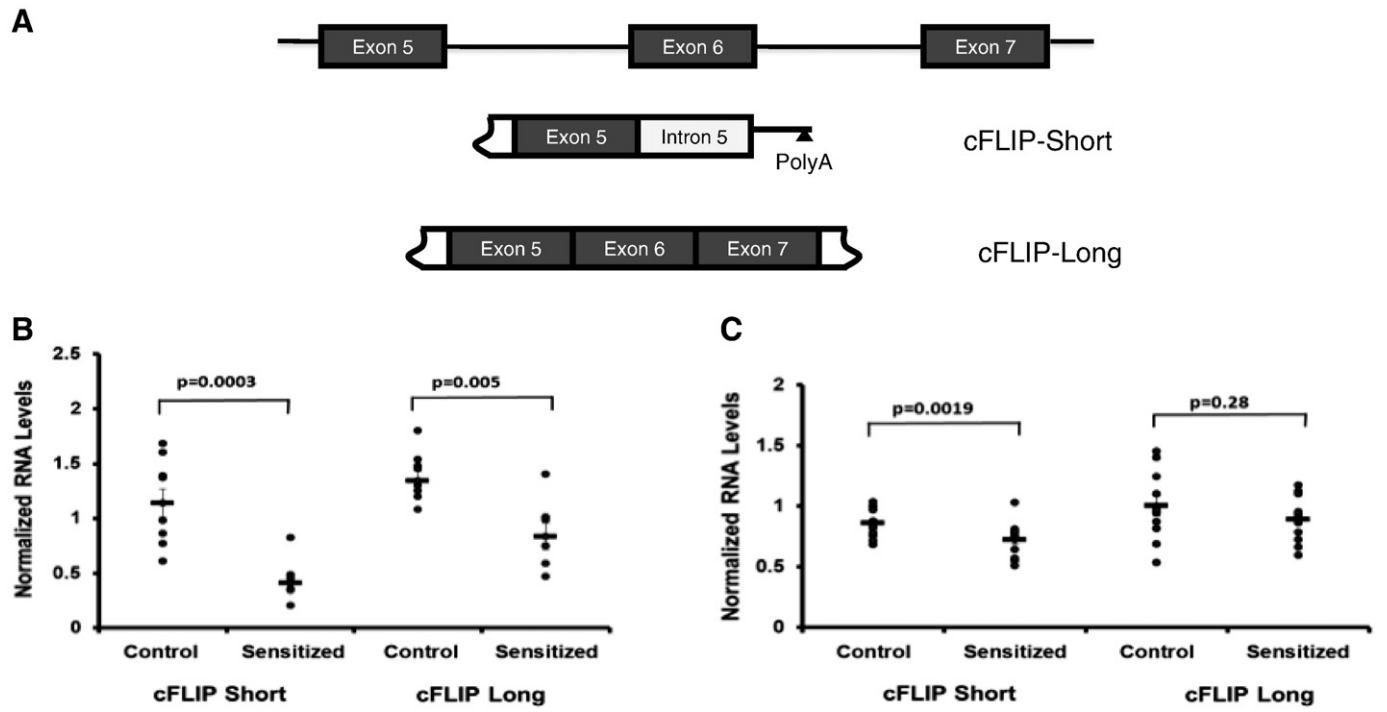

Fig. 2. *A. fumigatus* sensitization causes a shift alternative splicing pattern of murine cFLIP. (A). Genomic organization and mRNA isoforms of mouse cFLIP. The short isoform of cFLIP mRNA results in retention of a portion of intron 5 and subsequent utilization of the cleavage/polyadenylation site positioned in the intron. (B). Splicing microarray measurements of the relative levels of cFLIP mRNA isoforms in the spleen of control (n = 9) and *A. fumigatus* sensitized (n = 7) mice. For the purpose of this plot the signal strengths of all samples were normalized to an arbitrarily selected control sample. Horizontal bars represent average value for each set. (C). Quantitative RT-PCR measurements of the relative levels of cFLIP mRNA isoforms in the spleen of control (n = 11) and *A. fumigatus* sensitized (n = 12) mice. For each set of measurements the signal strengths were normalized to an arbitrarily selected sample. Horizontal bars represent average value for each set.

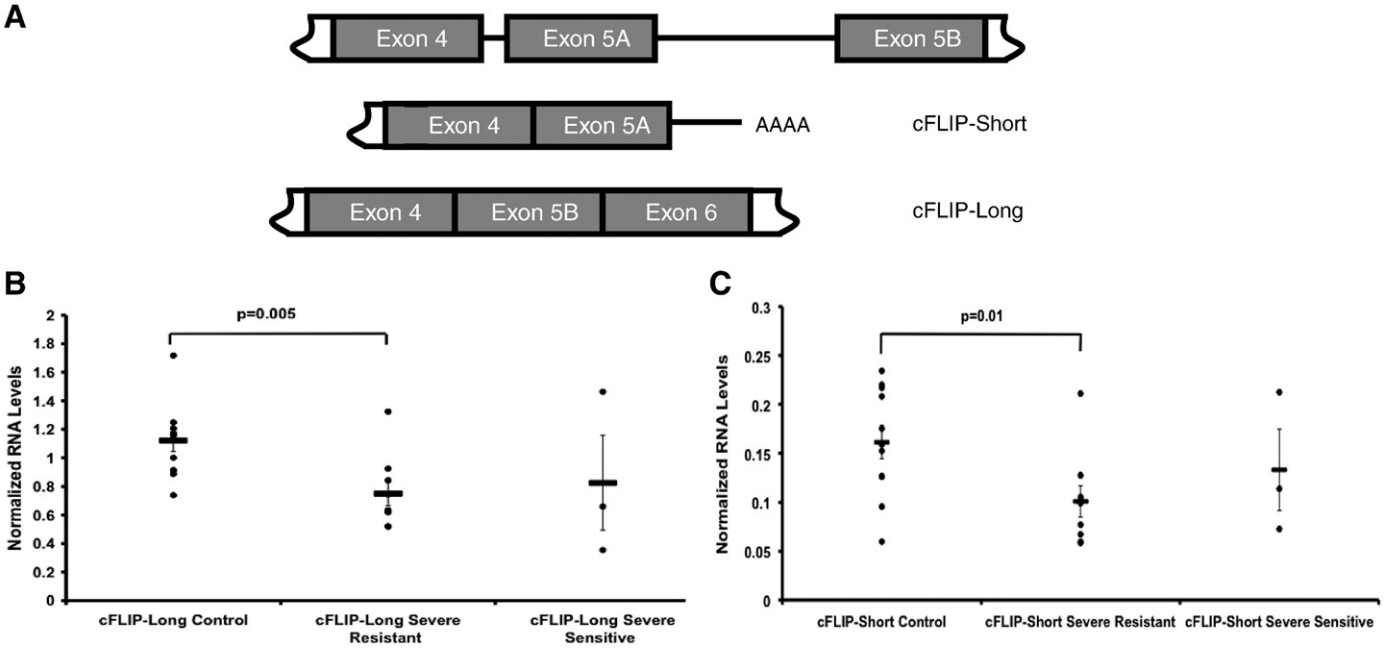

Fig. 3. Levels of both cFLIP mRNA isoforms are decreased in PBMCs severe corticosteroid resistant asthmatics. (A). Genomic organization and mRNA isoforms of human cFLIP. The short isoform of cFLIP mRNA results from splicing to a unique exon 5A and subsequent utilization of the cleavage/polyadenylation site positioned immediately downstream. (B). Quantitative RT-PCR measurements of the relative levels of the long cFLIP mRNA isoform in the PBMCs of healthy control subjects (n = 11), severe steroid sensitive (n = 3), and steroid resistant (n = 9) asthmatics. All sample signal strengths were normalized to an arbitrarily selected sample. Horizontal bars represent average value for each set. (C). Quantitative RT-PCR measurements of the relative levels of the short cFLIP mRNA isoform in the PBMCs of healthy control subjects (n = 11), severe steroid sensitive (n = 3), and steroid resistant (n = 9) asthmatics. All the signal strengths were normalized to an arbitrarily selected sample. Horizontal bars represent average value for each set.

cFLIP pre-mRNA is altered in human asthma, we used quantitative RT-PCR assays to measure levels of each isoform in RNA pools from PBMCs and neutrophils isolated from a group of 11 healthy individuals and a cohort of 12 severe asthmatics, which included 9 steroid-resistant and 3 steroid-sensitive patients. Importantly, we observed a statistically significant ~40% reduction in the levels of both $cFLIP_{Long}$ ($p = 0.005$) and $cFLIP_{Short}$ ($p = 0.01$) mRNA isoforms in PBMCs of severe steroid-resistant asthmatics when compared with healthy individuals (Fig. 3B, C). The levels of both isoforms also appeared to be reduced in the steroid-sensitive severe asthmatics, but the sample size was too small ($n = 3$) to draw definitive conclusions. Whether these results reflect changes in alternative splicing, differential stability and/or transcription is unclear, but they do indicate a reduction in cFlip mRNA in PBMCs isolated from severe asthmatics. There was no statistically significant asthma-dependent change in the levels of either isoform in neutrophils (data not shown).

SRSF2 (previously SC-35), a well-studied splicing regulatory protein belonging to the serine–arginine (SR) family of splicing regulators, has been implicated in regulation of $Cflip_{Short}$/$cFLIP_{Long}$ alternative splicing [33]. To investigate whether this protein plays a role in asthma-dependent changes in cFLIP expression, we measured the levels of SRSF2 mRNA in neutrophils and PBMCs of the patient cohorts described above. We observed no statistically significant changes in mRNA levels (data not shown).

Changes in relative levels of cFLIP isoforms have been shown previously to affect allergic airway sensitization in mice. Interestingly, overexpression of $cFLIP_{Long}$ in T-cells of transgenic mice was observed to lead to an intensified airway allergic and systemic response to ovalbumin challenge [41]. This increase in inflammation appears to stem from $cFLIP_{Long}$-associated induction of T helper (Th) 2 cytokines by both naïve and memory $CD4^+$ T cells accompanied by enhanced activity of cytokine transcription factor AP-1 and decrease of NF-kB activation. At present it is unclear whether this phenomenon is related to the effects of *A. fumigatus* sensitization on $cFLIP_{Long}$ expression. However, the possibility that the *A. fumigatus* sensitization-driven shift in cFLIP alternative splicing causes the above-mentioned effects on AP-1 and NF-kB is an intriguing possibility that merits further investigation.

Our results with the *A. fumigatus*-sensitized mice were on the one hand striking because they led to our discovery of a similar change in cFLIP mRNA levels in the PBMCs of severe, corticosteroid-resistant asthma patients. However, on the other hand, different mechanisms may underlie these changes. In mice the data are most consistent with a switch in alternative splicing, whereas the decreased levels of both cFLIP isoforms in humans may be due to a change in transcription or mRNA stability. While this discrepancy may reflect one of the limitations of using mice to represent human asthma (e.g., [42]), our findings also indicate the utility of the mouse model in studying splicing events and the discovery of novel pathways.

cFLIP is an inactive caspase-8 homolog that was described initially as a general inhibitor of the death ligand-mediated extrinsic apoptosis pathway. It is thought to act through sequestering procaspase-8 in CD95 death inducing signaling complex (DISC) through formation of cFLIP/caspase8 heterodimers (reviewed in [30]). An increase in cFLIP levels has been associated with reduction of apoptosis [14]. Later studies shaped a more nuanced model of cFLIP function that accounts for structural differences between its short and long protein isoforms. $cFLIP_{Long}$ was found to be able to activate procaspase-8 but not to cleave it, although this activation may be sufficient for apoptosis induction in certain conditions [6]. The $cFLIP_{Short}$ isoform lacks the caspase domain and thus, while it still sequesters procaspase-8, is unable to activate it. This isoform is associated strongly with inhibition of caspase-mediated apoptosis [5]. Its upregulation has been linked with resistance to activation-induced cell death [16], while downregulation of $cFLIP_S$ was shown to induce apoptosis [33].

Several studies have linked dysregulation of the extrinsic apoptosis pathway to asthma. Reduced sensitivity of airway lymphocytes to extrinsic apoptotic signaling [15,24] is believed to contribute to their prolonged survival [7,12], which, together with other factors such as eosinophilia [25], underlies chronic airway inflammation. In addition, mice that received Fas-deficient T-cells develop persistent airway inflammation, increased mucus production, and airway hyper-reactivity after antigen challenge [38].

Our results, however, appear inconsistent with previous observations of increased lymphocyte resistance to FAS-mediated apoptosis in asthma. Suppression of either or both isoforms of cFLIP would in fact likely lead to an increased susceptibility to extrinsic apoptotic signaling. There are several possible explanations for this apparent discrepancy. One is that increased resistance to apoptosis was observed primarily in airway T-cells [18,24], while we made our observations in PBMCs. Interestingly, Grzegorczyk et al. [11] observed that PBMCs isolated from allergic asthma patients undergo apoptosis more readily than PBMCs from healthy controls. Hence our results and their observation could potentially be explained by the differences between the landscapes of cytokine signaling in airway lumen and peripheral blood. Another possible explanation is that the observed change in cFLIP expression reflects activation of the normal anti-inflammatory signaling cascade that is blocked in severe asthma through an as yet unknown mechanism. There are some indications that an increase in severity of asthma may proceed in part through overwhelming the natural anti-inflammatory signaling through sustained pathological hyperstimulation. For example, peripheral blood leukocytes from patients with mild intermittent asthma displayed greater susceptibility to apoptosis than those isolated from patients with severe persistent asthma [1]. Alternately, this shift in cFLIP expression may represent an adaptive regulatory response aimed at resolving sustained inflammation through increasing the cells' susceptibility to apoptosis.

While many studies of dysregulation of the extrinsic apoptotic signaling in asthma have been conducted, its role in asthma development and/or asthma severity is still not fully understood. It is unclear, for example, whether breakdown of this pathway contributes to initial susceptibility to severe asthma, or is a consequence of chronic inflammation that results in severe symptoms. In this study we demonstrated that in asthma the function of this pathway may be affected through a decrease in mRNA levels of one of its intracellular components, cFLIP. Patterns of alternative splicing or reduced cFLIP mRNA isoforms may have important potential as biomarkers of severe inflammation and may serve as a potential target for therapy. Further research in this area has the potential to expand significantly our understanding of the physiological and genetic processes underlying this disease.

Materials and methods

Human subjects

Subjects with severe asthma as defined by National Asthma Education and Prevention Guidelines were screened for study participation [26]. Subjects with glucocorticoid resistant and corticosteroid sensitive asthma were enrolled. Glucocorticoid resistance was defined as daily asthma symptoms despite treatment with high dose inhaled corticosteroids (ICS: 1000 μg of fluticasone or its equivalent daily) or daily oral corticosteroids. Corticosteroid sensitive subjects were those with adequate asthma control (minimal symptoms with need for rescue bronchodilator less than 3 times per week) while on treatment with medium or high dose ICS (equivalent of 500 μg of fluticasone or more, see Supplementary Table 1). All subjects were being treated with at least one additional controller medication. All subjects were non-smokers with no history of smoking within the past year and had <10 pack per year history of smoking; all subjects were free of an asthma exacerbation for at least 4 weeks prior to volunteering for the study. Ten milliliters of blood was collected into a BD Vacutainer® CPT™ Cell Preparation Tube with sodium citrate (BD) according to the manufacturer's instructions. The PBMC layer was transferred into a

new 15-ml tube and PBS was added to the suspension to bring the total volume to 15 ml. The solution was mixed and recentrifuged at 1000 ×*g* for 10 min. The supernatant was removed and Trizol® was added directly to cell pellet. The study protocol was approved by the Columbia University IRB. All subjects signed informed consent.

Mouse samples

Six weeks old wild type female and male BALB/c mice were housed separately in the Columbia University animal facility and fed a commercial pellet mouse feed. Intranasal application of *A. fumigatus* (62.5 μg) in 50 μl of saline, or saline vehicle alone, was administered six times at four day intervals as described [19]. Subsequently mice were sacrificed and spleens were removed and cleared of fatty tissue. Spleens were washed three times with PBS, cut into 4–6 small pieces, one of which was transferred to a cell strainer. The spleen tissue was pulverized by pressing through the strainer mesh into a 50 ml tube with a syringe plunger. The strainer mesh was washed with 3–5 ml of PBS. Samples were centrifuged for 5 min at 1500 rpm at 4 °C. Pelleted spleen cells were suspended in 5 ml of RBC lysis buffer. Suspensions were incubated 10 min at RT (15–25 °C), and recentrifuged. Supernatants were discarded leaving behind pelleted leukocytes. Each spleen sample yielded $3.75 \cdot 10^6 - 4.5 \cdot 10^8$ cells/ml. All experimental procedures were approved by IACUC at Columbia University under protocols number AAAA7781 and AAAA3295.

RNA isolation

RNA was isolated from both mouse and human samples using TRIZOL® reagent (Invitrogen). For mouse splenic leukocytes, samples containing at least 10^7 cells/ml (nine control and seven sensitized samples) were used. Two millimeters of cell suspension was homogenized with 3 ml of TRIZOL® reagent. Insoluble material was removed by centrifugation, and RNA was extracted with chloroform and precipitated in isopropanol as per manufacturer's instructions. RNA was resuspended in 20 μl of DEPC-containing RNAse-free water. RNA quality was assessed by spectrophotometry and agarose gel electrophoresis (data not shown). Each sample yielded 3–16 μg RNA. Human PBMC RNA was prepared in essentially the same manner. Trizol® reagent (1–2 ml) was added directly to pelleted cells. Each sample yielded 80–180 μg total RNA.

Preparation of targets

Preparation of targets for microarray hybridization was done basically as described [8] RNA (1 μg per sample) was immobilized on streptavidin-coated magnetic beads using biotinylated oligo-dT and washed. Pooled query oligonucleotides were annealed to the RNA under a controlled hybridization program, and then washed to remove excess or mishybridized oligonucleotides. Hybridized oligonucleotides were then ligated to generate amplifiable templates. PCR was performed with fluorescently (Cy3) labeled universal PCR primers.

DNA microarray experiments

For microarray analysis, the Illumina Sentrix Expression BeadChip was used (Illumina, San Diego). Hybridization of labeled DNA (PCR products) to the BeadChip, washing and scanning was performed according to the Illumina BeadStation 500 X manual. Essentially the amplified DNA samples (9 control samples and 7 sensitized samples) were resuspended in a solution of Hyb E1 buffer (Illumina) and 25% (v/v) formamide at a final concentration of 25 ng/μl. 1.5 μg of each DNA was used for hybridization. Hybridization was at 55 °C for 18 h, after which the bead array matrix was washed for 10 min with 1 X high temperature buffer (Illumina), followed by a subsequent 10 minute wash in Wash E1BC buffer. Arrays were then washed with 100% ethanol for 10 min to strip off any remaining adhesive on the chip. A 2 minute E1BC wash was performed to remove residual ethanol. Arrays were blocked for 5 min with 1% (w/v) casein-PBS (Pierce) and signals were developed via 10 minute incubation with streptavidin-Cy3 at a final concentration of 1 mg/ml (GE Healthcare) in 1% casein-PBS blocking solution. The array was washed a final time in Wash E1BC buffer for 5 min and subsequently dried via centrifugation for 4 min at 275 rcf.

For analysis, arrays were scanned on the Illumina BeadArray Reader, a confocal-type imaging system with 532 (cye3) nm laser illumination. Image analysis and data extraction was carried out as previously reported [9]. The array images were registered using an algorithm described previously [10]. Essentially, bead signals were computed with weighted averages of pixel intensities and local background was subtracted. Each sequence-type signal was calculated by averaging corresponding bead signals with outliers removed (using median absolute deviation). Preliminary data analysis and QC was carried out using the BeadStudio software (Illumina). The raw data has been deposited at GEO under accession number GSE28258 in MIAME compliant format.

Microarray data analysis

The alternative splicing events represented on microarrays were initially filtered to eliminate those where at least one of the alternative mRNA isoforms yielded a signal intensity below 50 units in at least one of the 16 samples. Subsequently the mean signal intensity for each isoform was calculated separately for control and sensitized samples. Changes in each isoform's expression levels were assessed through calculating $\log_2$ of the ratio of mean intensity value of sensitized samples to mean intensity values of control samples. The significance of the observed changes was assessed through Student's two-sample unequal variance (heteroscedastic) t-test using two-tailed distribution.

Genuine changes in alternative splicing were subsequently identified by pair-wise examination of variations in the levels of mRNA isoforms that result from different possible outcomes of an alternative splicing event. Because authentic changes in alternative splicing would result in an overall change of ratio between two alternative mRNA isoforms, we used the following criteria to identify these events: First, we selected events where an at least 30% increase of one mRNA isoform was paralleled by at least 30% decrease of the alternative isoform. Second, we also selected events where at least a 2-fold increase or decrease of one mRNA isoform was accompanied by relatively stable levels of the alternative isoform (change less than 30%). Finally, we selected events where levels of both alternative isoforms either decreased or increased, but the degree of change differed significantly between the isoforms (at least 2-fold difference in the value of $\log_2$ of the ratio of mean intensity values of sensitized samples to mean intensity values of control samples). In all, 43 sets of isoforms met these conditions, and 16 of these displayed statistically significant changes (p-value <0.05 for both isoform levels between control and sensitized sample sets). Thirteen of these genes had homologous alternative splicing events in humans and were selected for further analyses.

Quantitative RT-PCR

500 ng of total human or mouse RNA was subjected to reverse transcription using a poly$(dT)_{16}$ primer and SuperscriptII® Reverse Transcriptase (Invitrogen) following the manufacturer's instructions. Subsequently the levels of both RNA isoforms were assessed in real-time PCR reactions using SYBR-Green (Fermentas®) and the 7300 Sequence Detection System (Applied Biosysems™). The correction factor used to compensate for the difference between template inputs between individual samples was determined in parallel reactions amplifying reference housekeeping genes: murine HMBS and HPRT with murine $cFLIP_{Short}$ and $cFLIP_{Long}$, respectively, and human SDHA and HPRT with human $cFLIP_{Short}$ and c-$FLIP_{Long}$, respectively. Both control and experimental groups in each species were assayed together in

duplicate in each experiment. After input correction, all samples (from both groups) were normalized to an arbitrarily selected control sample. Changes in expression levels of each isoform were assessed by comparing the average values for each group, and the p-values were determined using Student's t-test.

Declaration of conflict of interest

None.

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Appendix A. Supplementary data

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