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IL-32B is the predominant isoform expressed under inflammatory conditions *in vitro* and in COPD

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Abstract

Background: The IL-32 cytokine family is associated with chronic inflammatory diseases such as Chronic Obstructive Pulmonary Disease. However, this unique family is comprised of several alternative splicing isoforms and their expression pattern or responses to inflammatory stimuli are unclear. We aimed to determine which IL-32 isoforms are expressed in various cell types and in chronic airway disease, as well as their response to inflammatory stimuli.

Methods: We used several cell lines, human airway and lung primary cells from normal or chronic airway diseases to determine the basal expression of IL-32 isoform members and their response to inflammatory cytokine stimulations in cultured condition. We used real-time PCR to determine the gene expression of different IL-32 isoforms. We further applied hierarchical cluster analysis of the members of IL-32 based on their expression and response to inflammatory stimuli in different cell types. Finally, we analyzed IL-32 expression from published microarray data of different pulmonary diseases.

Results: By real-time PCR analysis, we found that IL-32B isoform expression is significantly higher in Chronic Obstructive Pulmonary Disease, but not asthma, compared to normal lungs. Analysis of basal expression of IL-32 isoforms revealed that cell lines from various lineages including NK cells, monocytes, epithelia, and human lung tissues have varying levels of IL-32 isoform expression. We also found that most cell types examined expressed IL-32B as the predominant isoform while peripheral blood mononuclear cells expressed IL-32A as the major isoform. Hierarchical clustering of IL-32 isoform based on responsiveness to inflammatory cytokines revealed that IL-32A, C, and D are regulated similarly in most of the cell lines when stimulated by TNF-α, IL-1β, and IL-6. By contrast, IL-32B and G did not share induction patterns with the other isoforms.

Conclusions: Our findings indicate that the specific isoform, IL-32B, but not pan-IL-32 isoforms, is elevated in Chronic Obstructive Pulmonary Disease, and that despite low basal expression, IL-32B is highly inducible by inflammatory cytokines in airway epithelial cells. The data also support that IL-32B is the predominant isoform and may be most relevant to lung inflammation.

Keywords: IL-32, IL-32 beta, Inflammation, Chronic obstructive pulmonary disease, COPD

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Background

Interleukin 32 (IL-32) is a recently described novel pro-inflammatory cytokine that does not belong to any of the known cytokine families [1]. It is implicated in chronic inflammatory diseases such as rheumatoid arthritis, Crohn's disease, ulcerative colitis, psoriasis, chronic obstructive pulmonary disease (COPD), atopic dermatitis and tuberculosis [2–7], as well as other diseases such as stomach cancer and in HIV replication [5, 8] (for review see [9]). Originally reported as NK transcript 4 (NK4) in activated T cells and IL-2 activated NK cells [10], NK4 was found to be elevated in peripheral blood mononuclear cells (PBMCs) from patients receiving high dose IL-2 therapy for metastatic melanoma [11]. Most notably, NK4 was identified as an IL-18-inducible gene in A549 lung epithelial cells transfected with the IL-18Rβ chain, and renamed "IL-32" due to its proinflammatory effects *in vitro* [1]. IL-32 is primarily expressed in immune cells, including mitogen-activated T cells, activated NK cells, and IFNγ-activated epithelial cells [12].

IL-32 is the product of a single gene, found on chromosome 16 (16p13.3) [1]. However, six alternative splice variants have been reported in various cell types [4, 13–17], although much work has not been done in this area to elucidate more definitive mechanisms of this potential mode of regulation. Using an adenoviral overexpression system, Henhuis *et al.* recently showed that IL-32G mRNA can be spliced into IL-32B, a less potent isoform [13]. Furthermore, IL-32D interacts with IL-32B and inhibits its function [18, 19]. These studies suggest a complex relationship between the IL-32 isoforms, and that IL-32B might serve as a modulator to dampen the strong proinflammatory effect of IL-32G. Although IL-32B is a less potent isoform than IL-32G, it is reported to be important in enhancing vascular inflammation and development of sepsis [20].

IL-32 sequences have not been found in the mouse genome; however, human IL-32 acts on murine cells, and transgenic mice carrying human IL-32 exhibit elevated inflammatory cytokine expression, more infiltrated leukocytes to tissues, and worsened sepsis, suggesting the presence of a receptor in the mouse that can respond to IL-32 [20]. However, no receptor for IL-32 has been identified to date, although proteinase 3 (PR3) has been identified as a soluble binding protein and while it can activate the functional activity of IL-32, the relationship and mechanism is unclear [21, 22].

As a proinflammatory cytokine, IL-32 has been reported to enhance inflammation. However, paradoxical regulatory functions have been described for IL-32. IL-32 has been found to induce inflammatory cytokine production in different cell types (Tumor Necrosis Factor (TNF)-α and IL-8 in THP-1 monocytes, IL-1β and IL-6 in PBMC, and MIP-2 in RAW macrophages [1]). Additionally, IL-32 enhances differentiation of monocytes into macrophages *in vitro* [23] and induces dendritic cells to direct Th1 and Th17 differentiation through the production of IL-6 and IL-12 [24]. However, IL-32 can also induce immunosuppressive cytokine IL-10 in murine models of Dextran Sodium Sulphate (DSS)-induced colitis, suggesting a regulatory role during the course of DSS-mediated inflammation [25]. Taken together, current evidence suggest that IL-32 is a proinflammatory cytokine, and effects downstream of IL-32 could be tightly regulated during the course of inflammation. Although there have been attempts at understanding the function of IL-32 *in vivo*, and the isoform expression in many cell types, it is still unclear which isoform of IL-32 is expressed in different diseases, and which is of higher biological significance. Here we have analyzed the expression patterns of IL-32 isoforms in lung disease tissues, in various cells lines and their inducibility to inflammatory cytokines.

Methods

Cell Culture, tissue and reagents

Wish (epithelial cell-like cell line originally derived from amnion epithelium), A549 (alveolar basal epithelial cell line), U937 (monocytic cell line), and primary Peripheral Blood Mononuclear Cells (PBMC) were cultured in 10 % FBS supplemented RPMI-1640 medium, and NK92MI (Natural Killer (NK) cell line) were cultured in the complete NK media (Medium base: α-MEM without ribonucleic acid, deoxyribonucleic acid, supplemented with 2 mM L-glutamine 1.5 g/L sodium bicarbonate, 0.2 mM inositol, 0.1 mM 2-mercaptoethanol, 0.02 mM folic acid, adjusted to 12.5 % horse serum and 12.5 % FBS). BEAS-2B (*B*ronchial *E*pithelial cells transformed by *A*d12-*S*V40 virus hybrid, Bronchial epithelial cell line) and NHBE (primary normal bronchial epithelia) cells were cultured in LHC-9 medium (Invitrogen, Inc.) in immersed culture conditions. The inflammatory cytokines: IL-1β, IL-6, TNF-α, IFNγ, IL-17A, TGFβ, IL-13 (R&D Systems), Lipopolysaccharide (LPS), and PMA/Ionomycin (Sigma) were used at the concentrations (ng/ml) indicated in the figures. Differentiated primary normal human bronchial epithelial cells (NHBE, from three healthy donors) cultured to form a three-dimensional model resembling the epithelial tissue of the respiratory tract were obtained from MatTek Corporation (Ashland, MA). Lung tissue samples from healthy ($n = 7$), COPD ($n = 7$) and asthma ($n = 4$) donors were obtained from Analytical Biological Services, Inc. (Wilmington, DE).

RT-PCR analysis

Total RNA was harvested from A549, Wish, NK92MI, U937, BEAS-2B, NHBE cells, and primary PBMC for

real-time PCR analysis (Roche). Primary airway tissues were homogenized using a homogenizer and total RNA extracted using RNA TRIzol reagent (Invitrogen, Inc., Carlsbad, CA). The expression of the five IL-32 isoforms in the untreated cells was determined by their relative gene expression to Glyceraldehyde-3-Phosphate Dehydrogenase (GAPDH) or β-actin (housekeeping gene) in each sample. Primers were ordered from Integrated DNA Technologies, Inc. (Coralville, IA), and had the following sequences:

NM_001012633.1:
hIL-32A QF1 CACCCAGAGCTCACTCCTCT
hIL-32A QR1 GGCTCCGTAGGACTTGTCAC
NM001012631:
hIL-32B QF1 GAAGACTGCGTGCAGAAGGT
hIL-32B QR1 CTTTCTATGGCCTGGTGCAT
NM001012634 and NM001012635:
hIL-32C QF1 AGGCCCGAATGGTGATGT
hIL-32C QR1 GGCACCGTAATCCATCTCTT
NM 001012636:
hIL-32D QF1 AGGACGTGGACAGGACGACT
hIL-32D QR1 AGGAGTGAGCTCTGGGTGCT
In UCSC genome database:
hIL-32G QF1 TACTTCTGCTCAGGGGTTGG
hIL-32G QR1 TGGGTGCTGCTCCTCATAAT

Microarray analysis

Publicly available microarray datasets were analyzed for the expression of IL-32 (GEO accession number GSE13896) [26]. As described in Shaykhiev et al., microarray was performed on mRNA collected from Alveolar Macrophages that were collected from bronchoalveolar lavage (BAL), followed by an adherence step resulting in >98 % Alveolar Macrophages [26].

Analysis of data and statistics

Analyses of data were accomplished using heatmap software in the program R (R Foundation for Statistical Computing). The clustering of samples and probes were performed using the hclust function. Briefly, the default setting used for this analysis employs the Euclidian distance measures between each objects using complete linkage method. The heat maps were drawn using the heatmap.2 function in the gplots library. Clustering of the IL-32 isoforms and treatments was based on log(fold induction), and clustering of basal expression was based on the value of (relative expression of IL-32 isoform/β-actin). The log (treated/untreated) determines the color of each treated sample. Red represents an increase in gene expression, while green represents a decrease in expression in the indicated samples. Data was also analyzed using GraphPad Prism® software (La Jolla, CA). Data are reported as average +/− SEM, with p values (derived from Student's *t*-test) given in the legend of the appropriate figures, and significance determined to be $p < 0.05$.

Results and discussion

Predominant expression of IL-32B in hematopoietic and epithelial cells

Due to the lack of reliable and specific antibodies able to detect individual IL-32 isoforms, we examined the expression of specific IL-32 isoforms in different human cell types by real-time PCR. We designed primers targeting specific IL-32 isoforms based on the NCBI and UCSC databases (Fig. 1). The IL32G mRNA sequence is not found in NCBI, so we used the published IL32G sequence from the UCSC genome bioinformatics database (http://genome.ucsc.edu/). The isoforms in the NCBI database are IL-32 alpha (A), IL-32 beta (B), IL-32 epsilon (C) and IL-32 delta (D), while the IL-32G sequence is available in UCSC database and published papers. There is a newer isoform, IL-32ζ, that has not yet been included in the NCBI database [17]. Here, we focused on the five major isoforms: IL-32A, B, C, D, and G, the gene structures of which are summarized in Fig. 1.

In this study, we tested the following primary cells and cell lines for IL-32 expression: NK92MI lymphoid cell line, PBMC, U937 monocyte/macrophage-like cells, Wish epithelial-like cells, A549 lung alveolar epithelial cells, BEAS-2B airway epithelial cells, and primary NHBE. First, we compared basal expression of the different IL-32 isoforms in these cell types (Fig. 2) and found that NK92MI, U937, and Wish cells have the highest expression of total IL-32, followed by A549. We detected minimal expression of IL-32 isoforms in PBMC, NHBE and BEAS-2B cells. Among the various isoforms, IL-32B was the dominant isoform expressed in NK92MI, U937, Wish, A549, BEAS-2B cells, and human lung tissues under basal (non-stimulated) conditions. By contrast, IL-32G was the highest expressed isoform in non-stimulated PBMCs, followed by IL-32B and IL-32A. We also examined primary human lung tissue and found that IL-32B was the isoform most highly expressed and IL-32A the lowest expressed isoform (Fig. 2).

IL-32 family responds poorly to several proinflammatory cytokines in NK92MI, U937, and Wish cells

We next determined the expression of IL-32 isoforms under various inflammatory stimulation conditions. IL-1β and TNF-α are two major cytokines that induce IL-32 gene expression [1, 27, 28], the latter via JNK and AKT pathways [29]. Therefore, we included these two cytokines as well as IFNγ, IL-6, IL-17A, LPS, and PMA/Ionomycin, to stimulate cells and analyze IL-32 isoform expression. The anti-inflammatory cytokine TGFβ, and Th2-associated cytokine, IL-13, were

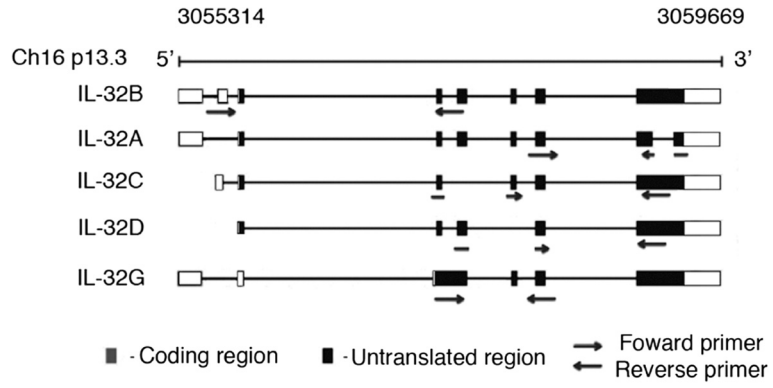

Fig. 1 Schematic of IL-32 isoforms as found in the NCBI database and primers used to analyze IL-32 isoforms A, B, C, D and G

included as well, given their role in asthma. Each cell type tested had a different pattern of response.

As shown in Fig. 3, stimulation of PBMCs with IL-1β, IL-6, TNF-α, LPS or PMA/Ionomycin led to increased expression of IL-32A, which becomes the dominant isoform after cytokine stimulation. Some induction of IL-32B is also seen by IL-1β, IL-6, and PMA/Ionomycin, but not by TNF-α or LPS (Fig. 3a). In the NK92MI cell line, all the isoforms are mildly induced by the proinflammatory cytokines IL-1β, IL-6 and TNF-α (Fig. 3b). In U937 cells, the IL-32B isoform is dominant and is only modestly induced by IL-1β (10 ng/ml). Although IL-32A and IL-32C are not the dominant isoforms, IL-32A can be induced by IL-6, TNF-α, and LPS, while IL-32C can be induced by IL-1β, TNF-α and LPS (Fig. 3c).

In Wish cells, IL-32B was the dominant isoform and was only induced two fold by TNF-α, while expression of IL-32A and IL-32G were low in non-stimulated conditions and significantly induced by TNF-α (Fig. 3d).

IL-32 family members are upregulated by several proinflammatory cytokines in airway and lung-derived epithelial cells: NHBE, BEAS-2B, and A549 cells

Overall IL-32 isoform expression is much lower in airway and lung-derived epithelial cells compared to NK92MI, U937, or Wish cell lines. However, the expression of IL-32 in these airway and lung-derived epithelial cells is highly inducible, with IL-32B induced by more than 100-fold upon IL-1β and TNF-α exposure in A549 cells (Fig. 3e). In BEAS-2B epithelial cells, IL-32B, C and G are also strongly induced by TNF-α. IL-1β and IFNγ also induced some IL-32 isoforms, IL-32A and IL-32D, but to a lesser extent (Fig. 3f). In NHBE cells, we also found that IL-1β, TNF-α, and IFN-γ significantly induce IL-32 isoforms (mainly IL-32B, C, and G). IL-6, IL-17A, and IL-13 did not have significant effects on isoform expression. Interestingly, IL-6 synergized with TGF-β to induce IL-32 isoform (Fig. 3g). Induction of all of the IL-32 isoforms was statistically significant (p *value* ≤ 0.05 by Student's t-test) unless indicated.

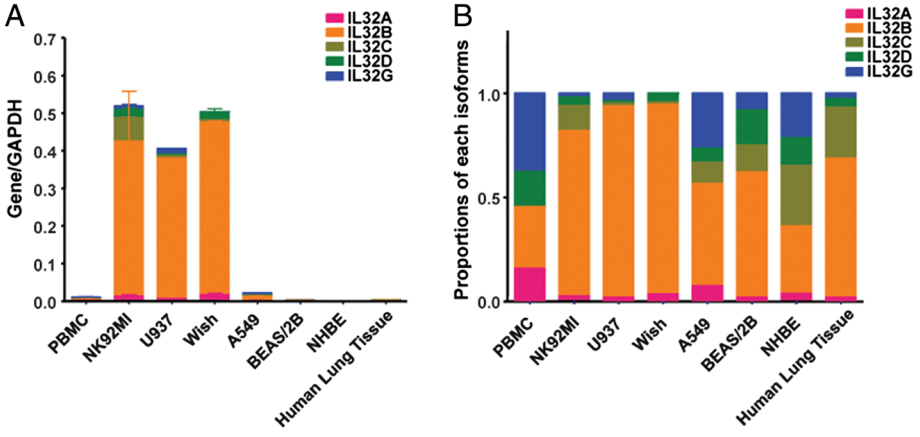

Fig. 2 Analysis of IL-32 isoform expression in the indicated cell lines. **a** Expression of IL-32 isoform in the indicated cell types as compared to the expression of the housekeeping gene GAPDH. **b** Expression of IL-32 isoform as a proportion of total IL-32 gene expression in the indicated cell types

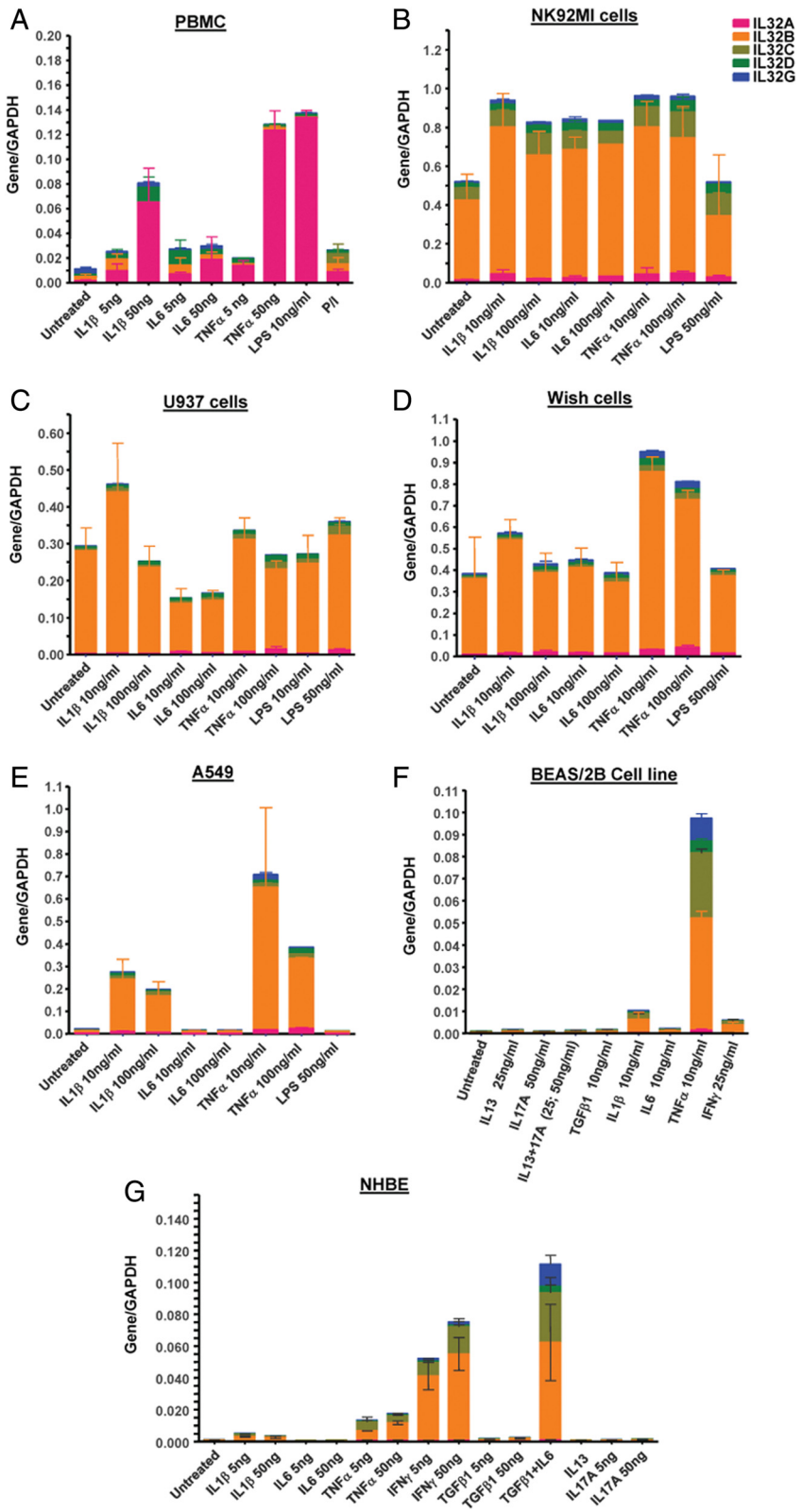

Fig. 3 Analysis of IL-32 isoform induction by inflammatory cytokines in the indicated cell lines. Expression of IL-32 isoform in the indicated cell types as compared to the expression of the housekeeping gene GAPDH following the indicated treatments. **a** PBMC. **b** NK92M1. **c** U937. **d** Wish. **e** A549. **f** BEAS/2B. **g** NHBE. Induction of all the IL-32 isoforms were statistically significant ($p < 0.05$ by Student's *t* test), unless indicated otherwise indicated

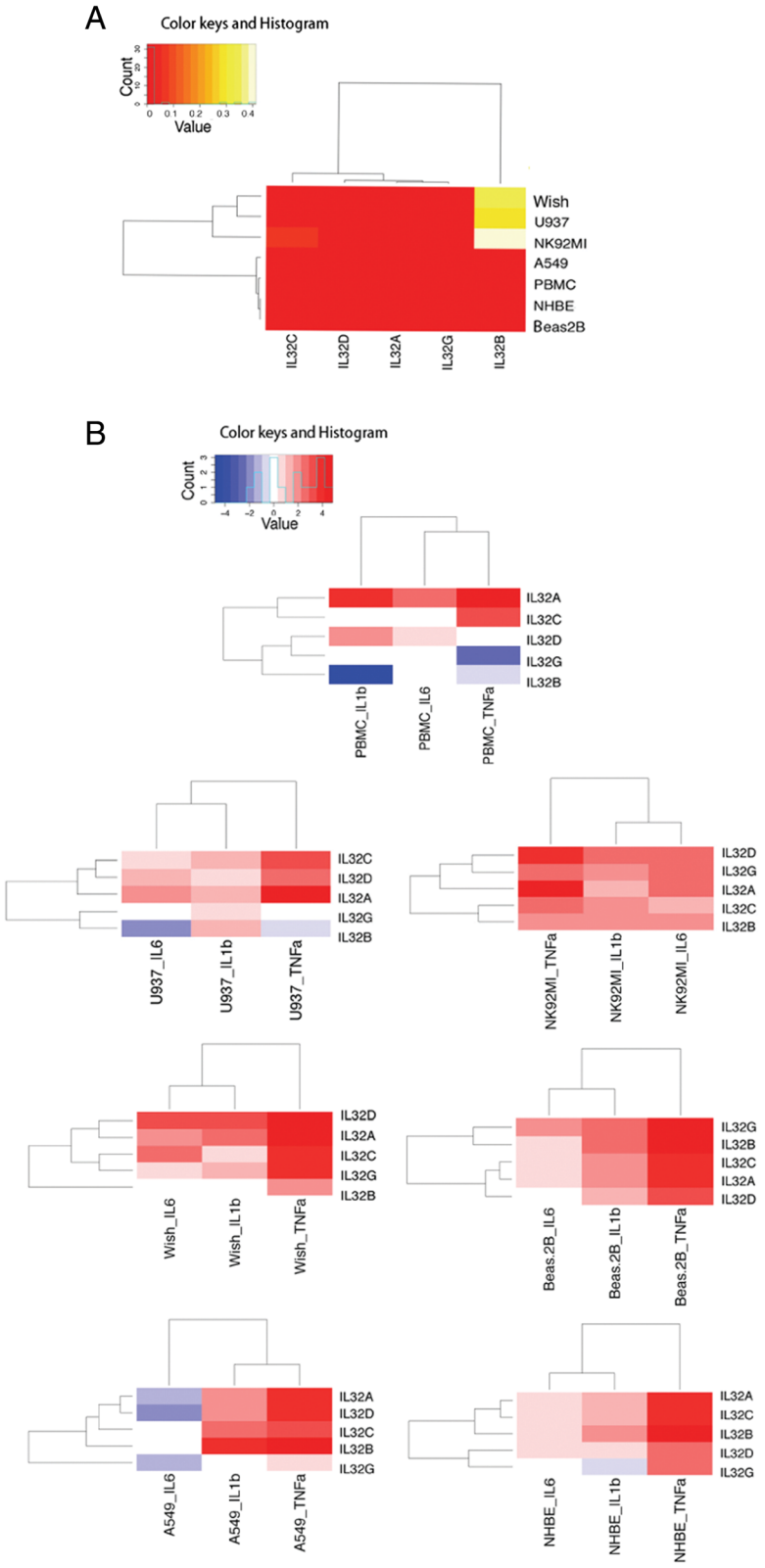

Fig. 4 (See legend on next page.)

(See figure on previous page.)
Fig. 4 Hierarchical clustering of IL-32 isoform induction by the indicated cytokines in the indicated cell lines. Expression of the indicated IL-32 isoform in the indicated cell types shown in Fig. 3 as analyzed by hierarchical clustering and heat map generation based on fold increase or decrease. **a** Clustering of the response of each isoform in each cell type based on the relative expression of IL-32 isoform/β-actin in the untreated condition. **b** Clustering of the data shown in Fig. 3 based on $\log_{10}$(fold induction) in response to the indicated cytokines. The log (treated/untreated) determines the color of each treated samples

Hierarchical cluster analysis of basal and inflammatory cytokine-stimulated IL-32 isoform expression in various cell types

By comparing the expression pattern of each IL-32 isoform with or without cytokine treatment, we can determine whether the induction of the different isoforms share similar pathways in different cell types. We therefore analyzed the data by hierarchical clustering and generated heat maps. Figure 4a shows the log of the fold-induction of each IL-32 isoform in each cell type as a heatmap and clustered based on their expression and induction level in specific cell lines. These analyses revealed that in U937, PBMC, NHBE, A549 and BEAS-2B cells, IL32A and IL32C behave similarly, suggesting these two isoforms may share common pathways of regulation in these cells. By contrast, IL32A and IL32D are more closely linked in Wish, A549 and NK92MI cell lines; and IL-32B and IL-32G are more closely linked in U937, PBMC, and BEAS-2B. In all cell types, IL-32B induction is not closely linked to IL-32A induction, and in most of the cells: Wish, U937, NK92MI, NHBE, A549 and BEAS-2B cells, IL-32B is not linked to IL-32D. This analysis reveals that induction of IL-32 isoform is uncoupled, dependent on the isoform being examined; IL-32B and IL-32G induction behaves differently from IL-32A, IL32C, and IL32D isoforms under TNF-α, IL-1β and IL-6 stimulation, while IL-32A, IL-32C and IL-32D behave more similarly in most of the cells upon stimulation by these cytokines.

The ratio of the basal expression of each IL-32 isoform to β-actin in each cell type were also plotted as heat maps and clustered based on their expression patterns (Fig. 4b). These analyses revealed that IL-32B is expressed at much higher levels than the other isoforms in Wish, NK92MI and U937 cell lines, but share comparable expression with other isoforms in BEAS-2B, A549, NHBE and PBMC cell lines. Note that the latter cell lines express lower levels of IL-32 in unstimulated conditions, suggesting that increases in expression of IL-32 is largely driven by increased expression of IL-32B.

Comparison of IL-32 gene expression in cells/tissues of airway disease by microarray analyses and isoform-specific real-time PCR

The expression of IL-32 has been implicated in a number of lung inflammatory diseases. We therefore reanalyzed microarray data of tissues from several airway inflammatory diseases obtained from NCBI public domain database. Comparing expression profiles in epithelial cells and alveolar macrophages from COPD and healthy controls, we found that IL-32 is upregulated in COPD epithelial cells compared to healthy/non-smoker ($p = 0.0156$ by Student's t-test), but down-regulated in COPD alveolar macrophages compared to healthy controls ($p = 0.0003$ by Student's t-test) (Fig. 5). By contrast, similar analysis of microarray data from airway epithelial cells of asthmatics, healthy controls, and smokers, revealed no difference of IL-32 gene expression between the three groups. The probes in microarray in these studies are pan-IL-32 probes.

To determine whether lungs from patients with chronic airway disease differentially express IL-32 isoforms, we examined frozen lung samples from different airway diseases by real-time PCR (Fig. 6a). We found that while overall IL-32 expression in COPD lungs was similar to that seen in normal lungs, IL-32B expression was significantly higher in lungs from COPD. However, IL-32C expression was higher in the normal lung compared to COPD lung. In contrast to COPD lungs, asthmatic lungs expressed relatively low levels of all isoforms of IL-32 tested compared to normal lungs. We also examined the expression of IL-1β and IL-6 in normal and COPD lung samples by real time quantitative PCR and found that IL-1β is significantly upregulated in COPD lungs, with a trend towards upregulation of IL-6 which did not reach statistical significance by Student's t-test (Fig. 6b,c).

IL-32 has been implicated in a number of inflammatory diseases. However, there are at least five different isoforms of this protein that can be expressed, and it is not clear which isoform is the most important on biological function. Previous analyses of the pattern of expression of this cytokine has either focused on IL-32G, or has not been clear as to which isoform was analyzed. In addition, analysis of the biological effects of IL-32 has focused on IL-32G [8, 10, 30, 31]. In this study, we examined the expression of the five IL-32 isoforms, IL-32A, B, C, D and G, in several cell types as well as in human lung tissues with airway diseases. We show that in the absence of stimulation, the B isoform of IL-32 is the most highly expressed in all the cell types tested in this study, with the exception of PBMCs. In addition, we show that the different isoforms are induced differentially by inflammatory cytokines, which may be

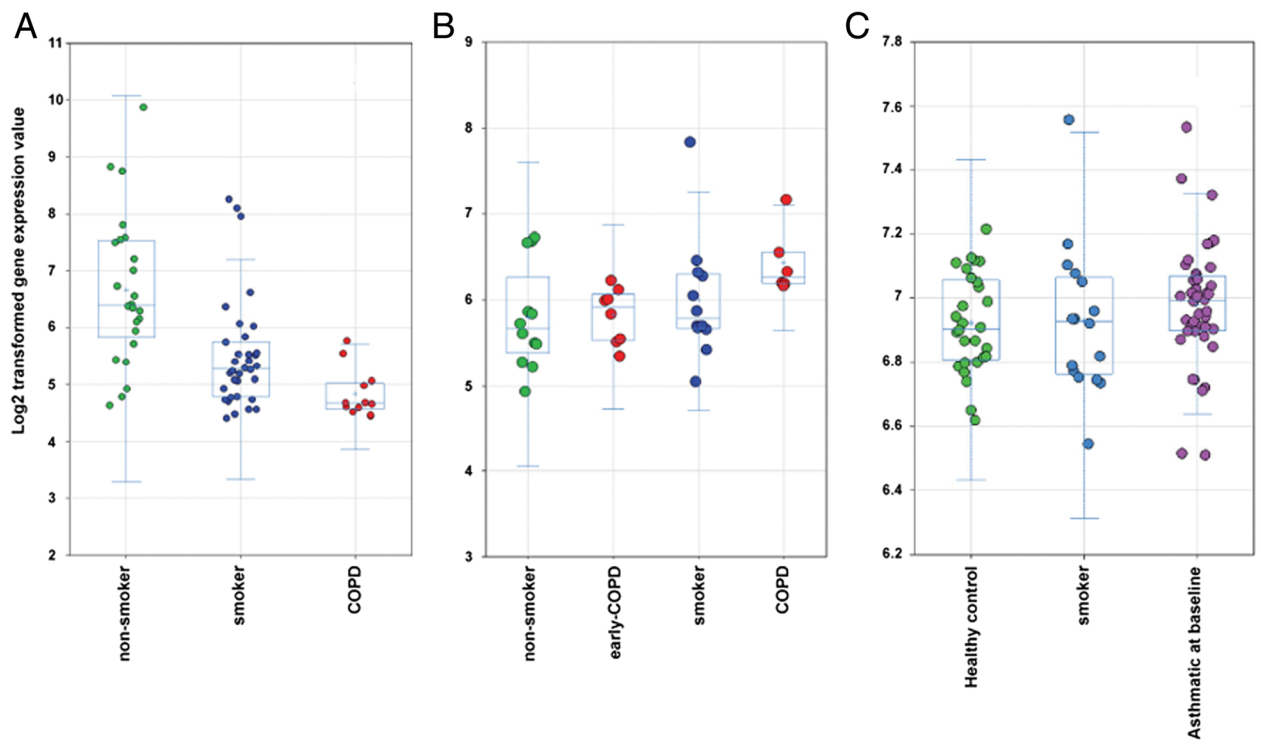

Fig. 5 Microarray analysis of IL-32 expression in cells from patients with the indicated disease. **a** Analysis of IL-32 gene expression in Alveolar Macrophages from healthy non-smokers ($n = 24$), healthy smokers ($n = 34$) and smokers with COPD ($n = 12$), non-smoker vs. COPD, $p = 0.0003$; non-smoker vs. smoker, $p = 0.0002$; smoker vs. COPD, not significant. **b** Analysis of IL-32 gene expression in Airway Epithelial Cells from non-smokers ($n = 12$), early COPD ($n = 9$), smokers ($n = 12$) and COPD ($n = 6$). non-smoker vs. COPD, $p = 0.0156$. Other comparisons are not statistically significant. **c** Analysis of IL-32 gene expression in Airway Epithelial Cells from normal donors ($n = 28$), smokers ($n = 16$) and asthmatic at baseline ($n = 42$). No significant differences observed

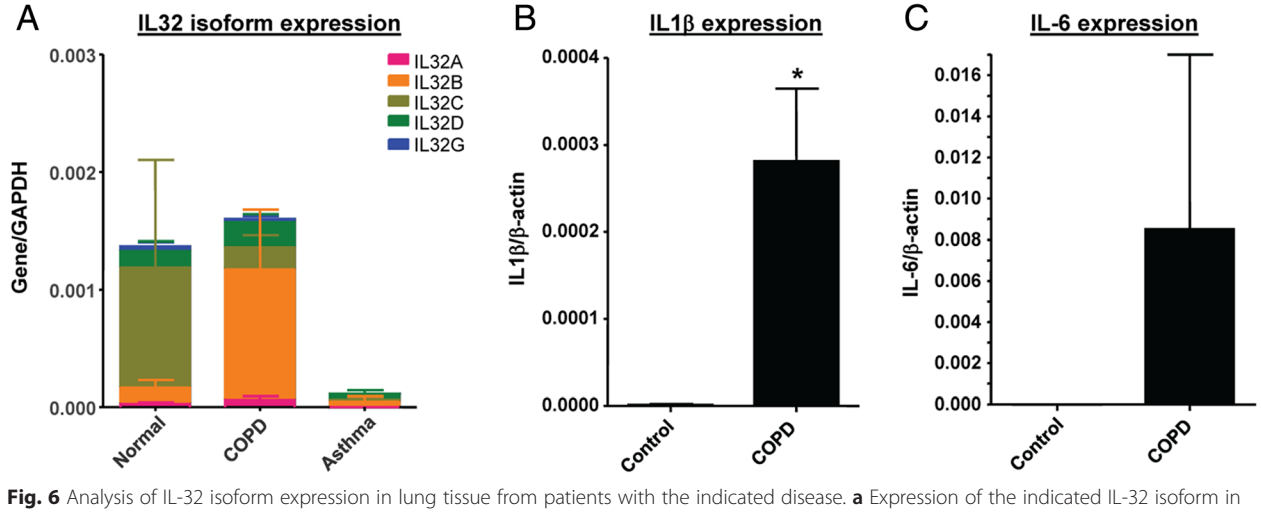

Fig. 6 Analysis of IL-32 isoform expression in lung tissue from patients with the indicated disease. **a** Expression of the indicated IL-32 isoform in the indicated lung samples was determined by RT-PCR. Normal ($n = 7$), COPD ($n = 7$), Asthma ($n = 4$). **b** Expression of IL-1β and **c**) IL-6 in normal and COPD lung samples was determined by RT-PCR. Normal ($n = 4$), COPD ($n = 3$).*$p < 0.05$ by Student's *t*-test

cell type specific. This induction of IL-32 was largely driven by increased expression of IL-32B in most cell types and to a lesser extent, IL-32G. Finally, our data indicates that while IL-32B isoform is highly expressed in COPD lung tissues, the total IL-32 isoform gene expression in COPD lung remains the same as normal lung tissues, suggesting that analysis of IL-32 isoforms may be more informative than total IL-32 expression.

Using isoform-specific PCR primers, we have characterized the response of IL-32 isoform mRNA expression in a variety of cell types to inflammatory stimulation. Our data indicate that depending on the origin of the cell type, different isoforms are induced preferentially. IL-32B is the predominant isoform in all the cell types tested in this study, with airway and lung-derived epithelial cells exhibiting the lowest basal level of IL-32 isoforms, but which were highly inducible. When treated with pro-inflammatory cytokines, leukocytic cells such as PBMC, NK92MI and U937 have predominant induction of IL-32A, while other cell types predominantly induced IL-32B in response to inflammatory cytokines. Further analysis of the behavior of these different isoforms under inducible conditions revealed that IL-32G and IL-32B behave similar, but differently from the other isoforms. Taken together with the finding that IL-32B can be generated from IL-32G mRNA [13], this suggests that IL-32G and B and may be linked in regulation, and perhaps together regulate inflammation. Our data suggest that IL-32 may be regulated not only at the transcriptional level, but also in alternative splicing, which seems to be cell type and stimulant specific.

Our analysis of the expression pattern of IL-32 isoforms indicate that while most studies have used IL-32G to examine the biological effects of IL-32, the predominant inducible isoform in leukocytic cells is IL-32A, and the predominant cytokine that is induced under inflammatory conditions in airway or lung-derived epithelial cells is IL-32B, although this may also be related to induction of IL-32G as discussed previously. More importantly, while the overall amount of IL-32 mRNA is the same in COPD lung tissues compared to normal ones, IL-32B is the predominant isoform that is significantly up-regulated in COPD lungs. Thus IL-32B may be a relevant biological isoform under inflammatory conditions, while IL-32A may be relevant in leukocytes. To determine whether different isoforms have different biological function, recombinant proteins from each isoforms, particularly IL-32B and IL32A, are needed. Recombinant IL-32G is commercially available, and we have attempted, with some success, to generate recombinant IL-32A protein. However attempts to generate recombinant IL-32B protein have been unsuccessful. As mentioned earlier, Heinhuis et al. recently reported that they were able to make IL-32B from IL-32G constructs due to the splicing of the IL-32G mRNA when expressed in cells. Using a mutant of the IL-32G mRNA that is unable to be spliced into IL-32B, they found that IL-32G had higher activity than IL-32B [13]. Developing IL-32 isoform-specific antibodies and recombinant IL-32 isoform proteins will be necessary to further study the differences in function of these spliced isoforms. With this limited availability of IL-32 recombinant isoform proteins, our preliminary data indicate that the biological responses to IL-32A and IL-32G isoforms are also dependent on the cell type being examined (data not shown).

Our analysis of normal bronchial epithelial cells in tissue culture conditions indicate that IL-32B is the dominant isoform under both non-cytokine stimulated and stimulated conditions. In addition, we tested IL-32 isoform regulation in human bronchial epithelial cells grown under air-liquid biphasic culture. Comparison of the two different culture conditions revealed no significant differences in IL-32 isoform expression between airway bronchial cells grown under immersed and air-liquid biphasic culture conditions. Furthermore, we compared the expression of IL-32 isoforms between normal and COPD donors grown under air-liquid biphasic culture and found that both normal and COPD bronchial cells have similar regulation of IL-32 isoforms in response to inflammatory cytokines (unpublished data). However, the challenges in obtaining sufficient numbers of these air-liquid interface cultured primary bronchial cells precluded us from making firm conclusions to determine if there is a difference in response to inflammatory cytokines in normal and COPD airways epithelial cells.

Our analysis of microarray data revealed that IL-32 expression is increased in COPD patients, with increases observed in epithelial cells, but not in lung alveolar macrophages. The probes from microarray data target the pan-IL-32 gene, therefore, information on expression of the individual IL-32 isoforms is limited in the microarray analysis. However, analysis of real-time PCR data of lung tissue from such patients reveal that the predominant isoform increased in COPD is IL-32B, while total IL-32 isoform expression in COPD lungs is similar to normal lungs. Indeed, Rong et al. recently reported that IL-32 protein is enhanced in cigarette smoke induced pulmonary inflammation [7]. By contrast, analysis of asthmatic lung and airway epithelial cells reveals that no significant increase in any individual IL-32 isoform. The results from our analysis of lung samples from asthmatics was unexpected since a recent study showed that a higher percentage of asthmatics than normals have detectable IL-32 in sera and sputum [32]. The discrepancy between our IL-32 expression data in lung/airway and their serum/sputum could be explained if there is tissue

specific expression of IL-32 expression in the asthmatics. Indeed, such tissue specific expression of IL-32 is found in COPD donors, as we have observed that while total IL-32 is increased in airway epithelial cells in COPD donors, it is not increased in our analysis of alveolar macrophage or lung tissues. It is also likely that different asthmatic populations, types of asthma and/or medication usage exhibit differential levels of IL-32 expression. Unfortunately given that we are blinded to our donors, we do not have this information to compare. As to the function of IL-32 in asthma, we found that IL-13, which can cause asthma exacerbation, does not increase any of the IL-32 isoforms in primary NHBE cultures. Meyer *et al.* found that IL-32 could inhibit angiogenesis, a process involved in airway remodeling. Interesting, they also observe further elevation of serum IL-32 protein after intensive asthma therapy. The elevation of IL-32 in allergic disease is not only observed in asthma, but also in atopic dermatitis where IL-32 plays a role in inducing apoptosis of keratinocytes [33]. Thus the expression of IL-32 may be regulated differentially in different tissues and during the pathological stages of asthma. Therefore, future experiments to elucidate the expression and function of IL-32 in different types and stages of asthma, as well as pre- and post-asthma therapy will be of great interest in this field.

Conclusions

Taken together, our data suggests that IL-32B is significantly upregulated in the inflammatory environment in COPD lungs and airway and lung epithelial cells. This suggests a potential role for the IL-32B isoform in pathological conditions in chronic lung diseases that are involved with proinflammatory cytokines, such as TNF-α, IL-1β, and IFNγ. Our hierarchical cluster analysis shows strong correlation between IL-32B and IL32G isoforms in terms of their responses to inflammatory cytokines, therefore it will be interesting to determine whether like IL-32G, IL-32B can further induce expression of inflammatory cytokines such as IL1β or TNF-α, creating a positive feed forward loop that contributes to the development of this disease, or whether IL-32B is a compensatory mechanism for the pro-inflammatory effects of IL-32G.

Abbreviations

COPD: Chronic obstructive pulmonary disease; PBMC: Peripheral blood mononuclear cells.

Competing interests

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Authors' contribution

Conception and design: FH, HL, SSJ, AA; Analysis and interpretation: FH, SW, HL, SSJ, AA; Drafting the manuscript for important intellectual content: FH, SW, HL, SSJ, AA. All authors read and approved the final manuscript.

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