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Collagen type-V is a danger signal associated with primary graft dysfunction in lung transplantation

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ABSTRACT

Background: Primary graft dysfunction (PGD) is the leading cause of early mortality after lung transplantation. Anti-collagen type-V (col(V)) immunity has been observed in animal models of ischemia-reperfusion injury (IRI) and in PGD. We hypothesized that collagen type-V is an innate danger signal contributing to PGD pathogenesis.
Methods: Anti-col(V) antibody production was detected by flow cytometric assay following cultures of murine CD19+ splenic cells with col.(V). Responding murine B cells were phenotyped using surface markers. RNA-Seq analysis was performed on murine CD19+ cells. Levels of anti-col(V) antibodies were measured in 188 recipients from the Lung Transplant Outcomes Group (LTOG) after transplantation.
Results: Col(V) induced rapid production of anti-col(V) antibodies from murine CD19+ B cells. Subtype analysis demonstrated innate B-1 B cells bound col.(V). Col(V) induced a specific transcriptional signature in CD19+ B cells with similarities to, yet distinct from, B cell receptor (BCR) stimulation. Rapid de novo production of anti-col(V) Abs was associated with an increased incidence of clinical PGD after lung transplant.
Conclusions: This study demonstrated that col.(V) is an rapidly recognized by B cells and has specific transcriptional signature. In lung transplants recipients the rapid seroconversion to anti-col(V) Ab is linked to increased risk of grade 3 PGD.

1. Introduction

Primary graft dysfunction (PGD), a form of acute lung injury occurring in the first 72 h post-transplant, is the leading cause of early mortality after lung transplantation [1]. Despite several limitations, multiple clinical risk factors have been associated with development of PGD [2]. However, the exact pathological mechanisms that lead to the development of PGD are not yet clear. There is strong evidence that lung auto-antibodies against self-antigens are also involved in development of PGD [3–5]. Humoral immunity against collagen type V collagen (col(V)) and k-alpha-1 tubulin (Kα1T), that are non-HLA antigens, predispose to the development of acute and chronic rejection in animal models and clinical transplant [4,6–9]. Col(V) is a minor extracellular matrix component in the lung where it is sequestered from the immune system by its location within the fibrils of collagen type I (col(I)), the major lung collagen [10]. However, due to the remodeling of the lung structure following ischemia-reperfusion injury (IRI), col.(V) becomes a target of cellular and humoral immune responses [4,11]. Previously, we have shown that col.(V) fragments were detected in the bronchoalveolar lavage (BAL) within 4 h post-transplant-induced IRI. More importantly, the passive transfer of anti-col(V) antibodies to rat lung isograft recipients reproduced the histology and pathophysiology of PGD [6]. Taken together these findings suggest that col.(V) may be a danger signal inducing rapid production of anti-col(V) Abs and implies

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the existence of innate B cells expressing anti-col(V) Abs [12].

In the present study, we evaluated the role of col.(V) as an innate danger signal for murine naïve B cells, their capacity to bind col.(V) on their cell-surface, and RNA profiles in B cells induced by col.(V). Furthermore we evaluated whether the rapid "de novo" production of anti-col(V) abs within 24 h after lung transplantation was associated with increased risk of PGD in small cohort of lung transplant patients.

2. Materials and methods

2.1. Animal studies

All mice were housed in the Laboratory Animal Resource Center at the Indiana University School of Medicine in accordance with institutional guidelines. All mice were 8 to 12 weeks of age and 24 to 32 g. All studies were approved by the Laboratory Animal Resource Center at the Indiana University School of Medicine. Specific pathogen–free male inbred mice C57BL/6 were purchased from Harlan Laboratories (Indianapolis, IN, USA).

2.2. Cell cultures

Unseparated splenocytes and CD19+ B cells (5×10^5/well) were cultured in complete medium including RPMI 1640 (Invitrogen) containing 10% heat-inactivated FBS (HyClone) and 1% penicillin/streptomycin, 1% glutamine (Invitrogen), in the presence of col.(I), col.(II), or col.(V) (each at 200 μg/ml), human vimentin (10 μg/ml) and Kα1T (1 μg/ml) in 96-well U-bottom plates for 72 h. For negative control, cells were cultured in complete RPMI 1640 with acetic acid in all the experiments. All cells were incubated at 37 °C, 5% CO_2 for the indicated times.

2.3. Flow cytometry detection of anti-col(V) Abs

Anti-col V Abs were detected in lung transplant patients' plasma and cell culture supernatants utilizing a patented flow cytometry bead assay (ImmuneWorks; Indianapolis, IN) [6]. All of the assays were run twice to validate the reproducibility to the assay and conducted as reported in prior publications [6,13]. Results of the duplicate reactions were expressed as Mean Fluorescent Intensity (MFI) as a fold change compared to control supernatants. A floating cut-off was determined by using a pooled plasma sample from 66 adult, non-smoking normal volunteers, as per published guidelines [14]. Samples were run in duplicate with percentage coefficient of variance (%CV) < 15. Longitudinal samples from each patient were assayed consecutively on the same plate.

2.4. CD19+ B cells immunophenotyping study by flow cytometry

Murine CD19+ B cells were isolated from the spleen and cultured in complete medium with col.(V). For negative control, CD19+ B cells were cultured with acetic acid. After 2 h, CD19+ B cells were collected, washed and stained with the following antibodies: phycoerythrin (PE)-conjugated anti-mouse immunoglobulin M (IgM), fluorescein isothiocynate (FITC)-conjugated anti-mouse CD21, PE-cy7 conjugated anti-mouse CD23 (eBioscience, clone II/41,4E3 and B3B4, respectively) and allophycocyanin (APC)- conjugated anti-col(V) Ab (Novus Biological, clone # NBPI-19633). Anti-col(V) Abs were labeled using lightning Link APC labelling kits (Innova Bioscience, Braham, UK) following manufacturers' guidelines. Stained cells were then analyzed on LSRII (Beckton Dickinson). Data were analyzed with FlowJo software (Treestar).

2.5. RNA isolation

Murine CD19+ (B cells) and CD19- (T cells) cells were separated using AutoMacs computer-controlled magnetic cell sorter (Miltenyi Biotec). Cells (1×10^6 ml) were then resuspended in 6 well plates in a total volume of 4 ml, and incubated for 3 h. Conditions included media, 5 mM HOAc, col.(I), col.(V), LPS, and $F(ab')_2$ anti-IgM. Purified col.(I) and col.(V) were soluble in 50 mM or 100 mM HOAC, respectively, so the culture media had 20 mM HEPES pH 7.5 added to restore the pH to neutrality. After 3 h, cells were centrifuged and cell pellet was lysed in 700 μL Trizol (Thermo-Fisher) for RNA preparation. A time zero sample was also collected and lysed in Trizol. Three independent experiments were completed and analyzed separately. RNA was purified using the Direct-Zol Mini Prep RNA Isolation Kit (Zymo R2052) with DNaseI on-column treatment. A group of genes from Fowler et al. were selected as markers of B cell activation [15]. cDNA was made from 1μg of total RNA using the High-Capacity cDNA Reserve Transcription Kit (Applied Biosystems 43,868,814) in the presence of Rnasin Plus Rnase Inhibitor (Promega N2611). Quantitative RT-PCR (qRT-PCR) was used to determine the level of mRNA expression of different genes selected to assess B cell purity, B cell activation status, and distinguish lipopolysaccharides (LPS) and IgM pathway stimulation based on Fowler et al. [15] qRT-PCR was normalized to the housekeeper gene mammalian (Mm) glyceraldehyde-3-phosphate dehydrogenase (Gapdh). Expression levels were determine by Ct value using Applied Biosystems 2× Taqman Assay Reaction Mix (4324018) on an Applied Biosystems Real Time 7500 instrument. Each biological replicate cDNA was run in duplicate. Statistical analysis used 2-way ANOVA and Tukey Test to assess differences between CD19+/CD19- isolated cell types and between various treatments of the CD19+ cells (GraphPad 7.0).

2.6. Cell separation purity

qRT-PCR analysis was used to determine cell separation purity. Several genes were identified as potential markers to distinguish CD19+ from CD19-: CD28, interlukin-4 (IL4) (T cell markers) and CD20 and immunoglobulin heavy constant mu (Ighm) (B cell markers). Cell separation purity was determined by looking at the ΔΔCt values of the CD19+ and CD19- populations at time zero (immediately after Gentle Macs cell separation), and after 3 h incubation with or without 5 mM Acetic Acid (HOAc). At time zero CD28 and IL4 had a 30-fold higher expression in the CD19- population, while CD20 and Ighm had a 10 to 15-fold higher expression in the CD19+ population, suggesting a high enrichment of CD19+ cells. After 3 h incubation with HOAC, expression of CD28, IL4, Ighm, and Myc were not affected, but CD20 expression decreased in both cell types while interferon regulatory factor 4 (Irf4) expression increased in only CD19+ cells.

2.7. Lung transplant patients

The Lung Transplant Outcomes Group (LTOG) cohort is a multicenter, prospective study that has been previously described [16]. We measured anti-col(V) Abs in 188 subjects transplanted between July 2007 and May 2010 with available plasma at all three of the pre-operative, 6 h (T6), and 24 h (T24) time points. Samples were collected from citrated tubes, processed within 1 h of collection and then stored at −80 °C for subsequent analysis. Clinical data were collected prospectively for all subjects as described previously [17]. Clinical characteristics are described in Table 1. Institutional review board (IRB) approval was obtained from each participating center and written informed consent was obtained from each subject enrolled in the cohort.

2.8. Determination of PGD grade

The primary outcome was grade 3 PGD within 72 h after transplantation, a definition that has demonstrated construct validity for mortality in prior publications [18]. PGD grade was determined using the ISHLT consensus definition [1]. Two blinded physicians examined chest radiographs to assess for the presence of PGD. The severity of PGD was graded according to the PaO2/FiO2 ratio, with a PaO2/FiO2

Table 1
Characteristics of the study population. Results are shown as number (n) or with median (IQR).

Characteristic	Col V cohort (n = 188)	LTOG total cohort (n = 1255)
Diagnosis		
ILD	68 (36%)	455 (36%)
COPD	60 (32%)	474 (38%)
CF	33 (18%)	178 (14%)
Other	27 (14%)	147 (12%)
Recipient race		
Caucasian	164 (87%)	1069 (85%)
Non-Caucasian	24 (13%)	185 (15%)
Female recipient	86 (46%)	546 (44%)
Recipient age	53 ± 13	54 ± 13
Recipient BMI	25 ± 5	25 ± 5
Pre-operative use of steroids	91 (48%)	604 (48%)
Donor race		
Caucasian	114 (61%)	802 (64%)
Non-Caucasian	68 (36%)	261 (21%)
Female donor	73 (39%)	493 (39%)
Donor age	34 ± 14	33 ± 14
Donor smoking	88 (47%)	479 (38%)
Bilateral transplant	133 (71%)	828 (66%)
Use of CBP	76 (40%)	466 (37%)
CBP TIME (min)	220 ± 70	223 ± 81
Ischemic time (min)	335 ± 101	318 ± 101
PASP (mmHg)	43 ± 17	43 ± 18
PRA class 1 (any)	43 (23%)	117 (9%)
PRA class 2 (any)	24 (13%)	77 (6%)

Abbreviations: ILD, interstitial lung disease; COPD, chronic obstructive pulmonary disease; CF, cystic fibrosis; BMI, body mass index; CBP, cardiopulmonary bypass; PASP, pulmonary artery systolic pressure; PRA, panel reactive antibody, measured prior to transplant.

ratio < 200 defining grade 3 PGD [19].

2.9. Statistical analysis

Data generated from animal experiments are expressed as mean ± SEM. Analysis was by either 2-way ANOVA with paired or non-parametric *t*-test using Prism 4 (GraphPad Software, San Diego, CA). Significance was determined by $p < .05$. Levels of antibodies against col.(V) in lung transplants patients were analyzed as a continuous variable with calculation of longitudinal percent of change. A Kruskal-Wallis H test was used to evaluate incidence of grade 3 PGD in 4 different groups. *P* values of less than or equal to 0.05 were considered significant. Analyses were performed using STATA version 14.0 (STATA Corp., College Station, TX).

3. Results

3.1. Production of anti-col(V) antibodies is contact- and time-dependent and specific to col(V)

To determine whether col.(V) induced the production of anti-col(V) Abs and if this process was contact dependent, murine splenocytes were cultured in the presence of col.(V) directly or separated via a trans-well permeable membrane and Abs production was detected in culture supernatants as described on the online data supplement. Col(V) induced contact-dependent anti-col(V) Abs production (Fig. 1A). To determine whether this was due to a direct effect on B cells, as well as the total time to Abs production, purified CD19+ splenic B cells were cultured with col.(V) for varying time periods. Col(V) induced anti-col(V) time-dependent Abs production significantly at 72 h as compared to 24 h (Fig. 1B).

Next, we asked whether the production of anti-col(V) Abs was col.(V) antigen-specific. Murine splenocytes and CD19+ B cells were cultured with col.(I), the major lung collagen [20]; collagen type II (col (II)) which is present in articular cartilage, but not the lung [21]; Kα1T, an autoantigen implicated in lung allograft rejection [9]; and vimentin, an autoantigen associated with the pathogenesis of several autoimmune syndromes as well as rejection of solid organ transplants [22]. Anti-col (V) Abs production was measured in culture supernatants after 72 h. Col(V), but not other antigens, induced production of anti-col(V) Abs from splenocytes or CD19+ B cells (Fig. 1C and D).

3.2. B1−B cells presented significantly higher frequency of surface col(V) binding

Since innate B cells (B-1 B, IgM+ $CD23^{low}$ $CD21^{low}$) can be the source of rapid Ab response [23], we next determined if this B cell subset bound col.(V). Follicular B cells (FO), B1−B cells (B1), and Marginal Zone B cells (MZ) populations were distinguished by flow cytometry (Fig. 2A). B-1 B cells demonstrated the highest frequency of col.(V)-binding cells compared to MZ B cells and FO B cells ($p \leq .001$) (Fig. 2B).

3.3. Col(V) induces a specific transcriptional pathway on murine CD19+ B cells

We then assessed the transcriptional profile induced by col.(V). As shown in supplemental fig. 2, LPS or BCR stimulation of mouse splenic CD19+ cells produced broad transcriptional responses within 3 h of treatment compared with no treatment or control, HOAc. Using a false discovery rate (FDR) cut-off q-value < 0.001, there were 3676 differentially regulated genes in both LPS and BCR stimulation groups (Excel File S1). A fold-change scatterplot comparing LPS vs BCR stimulation demonstrated a significant positive correlation in differential gene expression between the two treatments (r = +0.766; *p*-value < 1xE-06; Fig. S2), similar to that shown by Fowler et al. [15]. All but 333 genes were concordantly regulated (i.e., both up/up or both down/down), giving rise to the strong positive correlation in differential gene expression.

In order to focus on the more subtle response of the murine CD19+ cells to collagens, the analysis was repeated using just the HOAc negative control, BCR stimulation, col.(I), and col.(V) treatments. Principal component analysis (PCA) to visualize overall relatedness of different datasets (Fig. 3A) demonstrated that col.(I) treatment did not induce a discernable response relative to the HOAc negative control. Col(V) and BCR each had distinguishable responses from each other as well controls (Fig. 3A), though (Fig. S3) the col.(V) effects were more similar to BCR than to LPS responses. The subset of discordantly regulated genes (n = 333; Fig. S4, Excel File S1) was used as a transcription profile fingerprint to test whether col.(I) or col.(V) treatment produces a transcriptional response similar to LPS or BCR stimulation of CD19+ cells. Scatterplots were produced comparing the gene expression of this subset of genes of LPS or BCR stimulation with col.(I) or col.(V) treatment. The gene expression pattern after col.(V) treatment showed a significant positive correlated to BCR stimulation (Fig. S5); col.(V) vs BCR r = +0.492 (correlation *p*-value < 1xE-05), quadrant count Fisher Exact test < 1xE-08), while col.(I) treatment did not (col(I) vs BCR $r = -0.099$, Fisher Exact test = 0.70).

Pathway analysis of the col.(V) differentially expressed (DE) genes (n = 462; q < 0.001) was performed using Enrichr [24], focusing on the MGI's Mammalian Phenotype (MP) database [25], and commercially available Ingenuity Pathway Analysis™ (IPA), focusing on the Upstream Regulators, and predicted network analyses (Fig. 3B). There were 26 top scoring MP terms (adjusted p-value < .01; combined score > 10) of which 13 were specific to col.(V) DE genes; 10 were in common with BCR DE genes, while on 3 were shared between col.(V), BCR, and LPS treatment DE genes. There were no statistically significant MP terms identified using the col.(I) DE gene list. The vast majority of the MP terms were related to immunological responsiveness. The 32 potential upstream gene regulators identified by IPA from

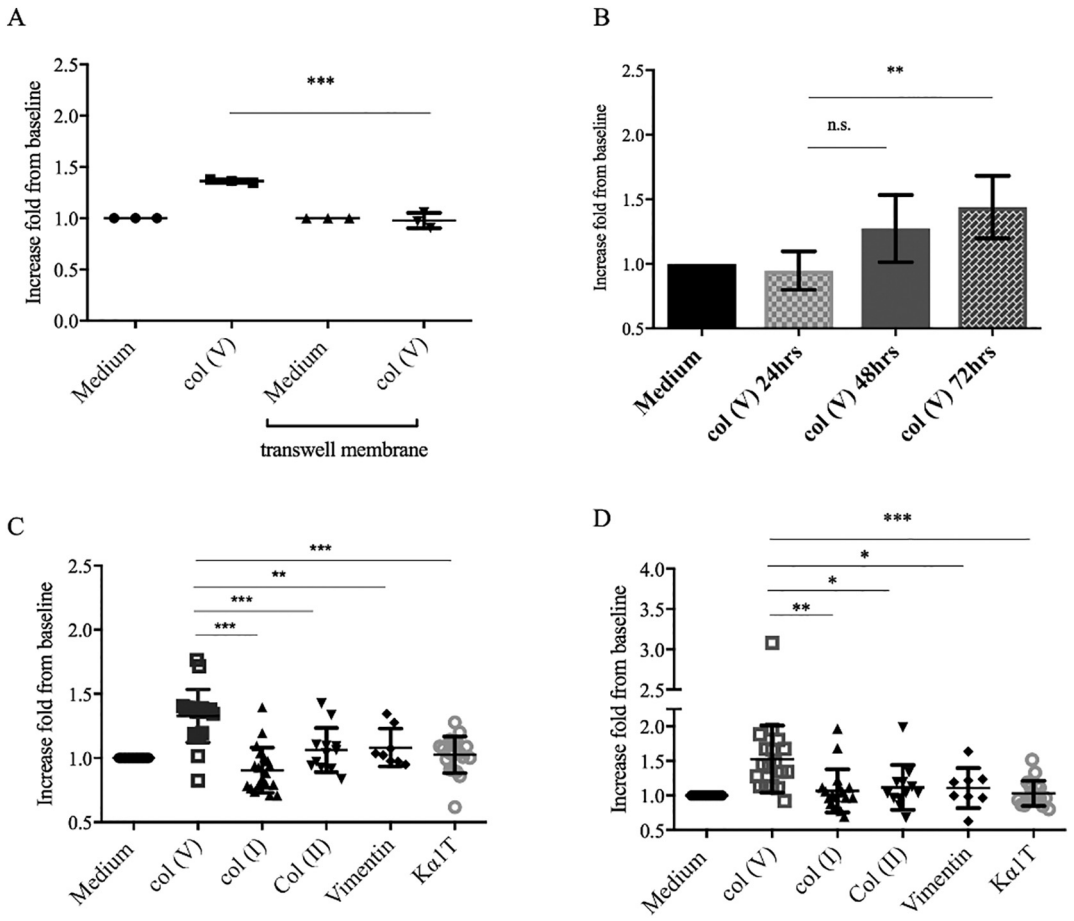

Fig. 1. Production of anti-col(V) antibodies is contact- and time-dependent and specific to col(V). Flow cytometric analysis of murine naïve splenocytes and CD19+ B cells supernatants for the production of anti-col(V) antibodies. (A) Murine naïve splenocytes were in culture for 48 h with col.(V) or separated by a transwell permeable membrane. The presence of a transwell membrane significantly abrogated the production of anti-col(V) Abs. (B) Naïve CD19+ B cells in culture with col.(V) at 24, 48 and 72 h. Significant increase in anti-col(V) Abs production at 72 h when compared to 24 h. $N = 3$ to 4 per condition. (C) Murine naïve splenocytes and (D) CD19+ B cells were stimulated for 72 h with of col.(V), col.(I), col.(II), human vimentin or Kα1T, respectively. Data generated from animal experiments ($N = 20$) are expressed as mean ± SEM. Statistical testing included unpaired *t*-tests and analysis of variance. Results of the duplicate reactions were expressed as Mean Fluorescent Intensity (MFI) as a fold change compared to control supernatants (*** $P \leq .001$, ** $P \leq .01$, * $P \leq .05$).

the col.(V) DE gene list were also focused on immunological pathways, including interferon-γ (Infg), tumor necrosis factor (Tnf), transforming growth factor beta 1 (Tgfb1), interleukins, signal transducers and activators of transcription, and TLRs. Interestingly, 12 of these potential regulators were predicted to lead to activation of their pathways (predicted z score > +2.0) while only 1 (interleukin 1 beta (IL1b)) was specifically identified as a DE gene. The top-ranked network assembled by IPA (consistency score = 34.6; equivalent to a Fisher Exact test *p*-value < 1xE-34) from the col.(V) DE genelist linked 13 potential upstream regulators with a wide network of potential target genes (Fig. 3B). From modeling this predicted network, the upstream regulators included C−C motif chemokine ligand 2 (CCL2), interleukin 32 (IL32), interferon regulatory factor 6 (Irf6), lymphotoxin α (Lta), nuclear factor kappa B 1 (Nfkb1), and signal transducer and activator of transcription 5a/5b (Stat5a/5b).

Total RNA from CD19+ B cells was processed and analyzed using Illumina sequencing methods and a standard RNA-seq processing pipeline. Principal component analysis after batch correction shows good separation of reflection col.(V) induced transcriptional changes relative to controls and BCR activation. (B) The col.(V) differentially expressed (DE) genes ($n = 462$; q < 0.001) were imported into Ingenuity Pathway Analysis™ and the top-ranked network assembled (consistency score = 34.6; equivalent to a Fisher Exact test *p*-value < 1xE-34) is shown.

3.4. Rapid de novo anti-col(V) Abs production is associated with severe PGD in lung transplant patients

We then sought to determine whether seroconversion of anti-col(V) Abs was associated with development of severe grade 3 PGD after lung transplantation. Anti-col(V) Abs were assayed pre-transplant and post-allograft reperfusion at 6 and 24 h in the blood of 188 lung transplant patients from Lung Transplant Outcome Group (LTOG) cohort [1]. Of those, 78 (41%) experienced a grade 3 PGD within 72 h from time of reperfusion. There were not significant differences in demographics between the population used to measure col.(V) and the greater LTOG cohort (Table 1). We identified four groups of patients based on anti-col (V) Abs assay: (1) no seroconversion ($n = 134$); (2) persistent positive anti-col(V) Abs pre- and post-transplant ($n = 23$); (3) positive to negative anti-col(V) Abs post-transplant (n = 13); and (4) seroconversion from negative to positive anti-col(V) Abs within 24 h ($n = 8$). The 8 patients, who demonstrated seroconversion from negative to positive anti-col(V) Abs within 24 h, developed severe grade 3 PGD post transplant. A Kruskal-Wallis H test showed that there was a statistically significant incidence of grade 3 PGD post-transplant ($\chi^2(3) = 10.725$, $p = .01$) after seroconversion to positive anti-col(V) Abs (Fig. 4).

4. Discussion

The key finding of the present study is that col.(V) is a novel danger

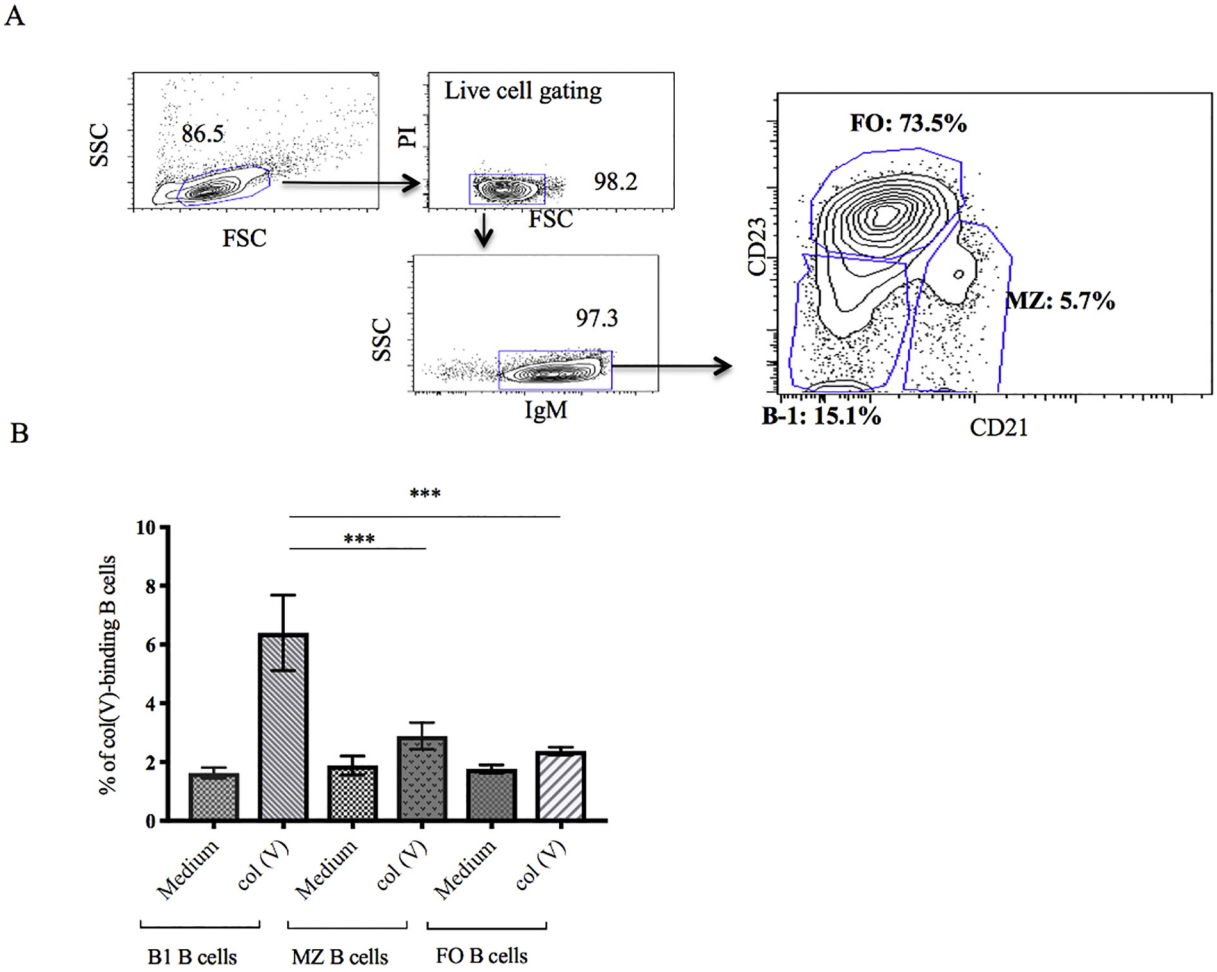

Fig. 2. B-1 B cells present higher frequency of col(V)-binding B cells on the cells surface. (A) Murine CD19+ B cells were cultured for 2 h in complete medium with col.(V). For negative control, CD19+ B cells were cultured with acetic acid. CD19+ cells were identified among total lymphocytes (SSC vs FSC). The lymphocyte gate was further analyzed for their uptake of Live/Dead stain using propidium iodide vs FSC. B cell subsets were identified initially by their expression of IgM then further gated on their expression of CD21 and CD23: Follicular B Cells (FO) (IgM+, CD23high, CD21neg), B1−B cells (B1) (IgM+, CD23low, CD21low), Marginal Zone B cells (MZ) (IgM+, CD23low, CD21+). (B) Percentage of col.(V)-binding B cell subsets after 2 h stimulation with col.(V). Stained cells were then analyzed on LSRII (Beckton Dickinson). Data were analyzed with FlowJo software (Treestar). $N = 5$ per condition. *** $P \leq .001$.

signal that induces rapid anti-col(V) humoral response from B1−B cells; moreover, this "de novo" humoral response is clinically associated with the development of PGD in lung transplant patients.

The presence non-HLA antibodies have been described in up to 33% of patients listed for lung transplant and up to 70% of recipients following lung transplantation [3,26]. The presence of non-HLA antibodies against col.(V) and Kα1T before transplantation is associated with increased risk of developing PGD in lung transplant recipients [3,9]. Both these self-antigens are expressed in the lung small airways. Col(V) is a minor extracellular matrix component, while Kα1T is a gap junction protein [27]. In animal models, our lab firstly demonstrated that passive transfer of anti-col(V) antibodies induced impaired oxygenation, complement dependent antibody cellular cytotoxicity in airway epithelial cells, and acute lung injury compatible with PGD [6]. In the present study, we showed a significant increase in anti-col(V) Abs at 72 h suggesting that the CD19+ B cells are innately primed to recognize col.(V) and to produce natural antibodies, which can induce pathological consequences in specific clinical scenarios. We were not able to identify a definite col.(V) receptor on murine B cells. Further studies are necessary to identify a specific receptor for col.(V), including collagen-binding receptors such integrins but also other members of the Tlr system as TLR9.

The RNA-seq experiments demonstrated a unique CD19+ cell transcriptional response to col. (V) treatment. Using the discordantly regulated genes derived from comparing LPS vs. BCR transcriptional programs, the col.(V) transcriptional response was more similar to BCR treatment, suggesting col.(V) may be activating a receptor signaling pathway. The upstream regulator predictions from IPA identified Tlr3, 4, and 9 as statistically enriched, of which only Tlr9 pathway was indicated to be activated, perhaps indicating this as a mechanism regulating col.(V) transcriptional response. A gene network from the col.(V) DE gene list was assembled by IPA (Fig. 3C), pointing to the potential involvement of Ccl2, Il32, Irf6, Lta, Nfkb1, and Stat5a/5b, all of which have been identified in the literature of transplant rejection [28].

PGD represents a severe clinical syndrome that develops in the first 72 h following lung transplantation and it is characterized by damage to pulmonary endothelium and epithelium [29]. Despite several clinical risk factors have been identified, the underlying pathological mechanisms that lead to the development of PGD are still unclear. Multiple studies suggest that autoimmunity is playing an emerging role in the development of this form of lung injury after transplantation [5,9]. Bharat et al. described the increased risk of developing PGD in lung transplant recipients with pre-transplant self-antibodies. Pre-transplant antibodies against col.(I), col.(V) and Kα1T were found in 41 patients out of 142 lung transplant recipients. The risk of developing PGD was higher in patients with all three antibodies present [9]. In the following study including a larger cohort of lung transplant recipients, the presence of antibodies against self-antigens col.(V) and Kα1T before

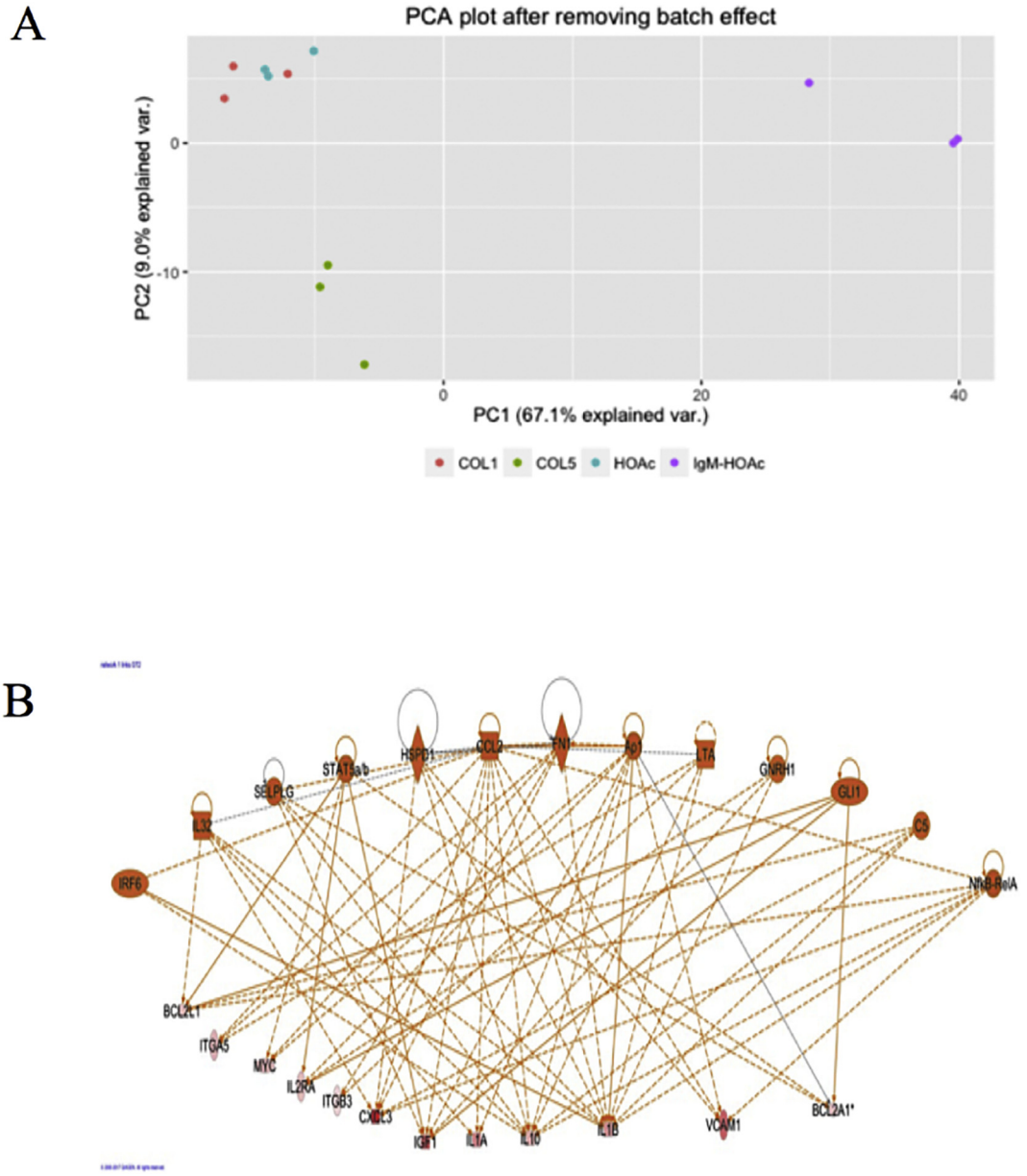

Fig. 3. RNA-seq analysis of col.(V) specific transcriptional program.

transplantation was associated with increased risk of developing PGD [3]. Despite both studies are limited by analyzing only pre-transplant samples without serial analysis after transplantation, they provided strong evidence of the participation of self-antigens in the development of PGD. While the ability of Kα1T antibodies to reproduce PGD in animal models has not been determined, the passive transfer of purified anti-col(V) antibodies to rat lung isograft recipients reproduced the histology and pathophysiology of PGD [6]. Moreover, as demonstrated by Iwata et al. col.(V) fragments are detectable in the BAL as soon as 4 h after lung transplantation in animal models [12]. Subramian et al. showed in their mouse orthotopic left lung transplant model the presence of col.(V) antibodies as early as 7 days after transplantation. These findings support the hypothesis that humoral immune response against col.(V) antibodies could potentially be rapid and harmful following transplantation in the setting of a highly inflammatory condition. Working on this hypothesis, for the first time we measured the presence of anti-col(V) antibodies before and after lung transplantation in a small cohort of lung transplant recipients. The most interesting finding was that the appearance of de novo anti-col(V) Abs within the first 24 h after organ reperfusion is associated with an increased incidence of grade 3 PGD, even though only a small number of patients seroconverted. This result is particularly interesting due to the prompt production of humoral immunity against col.(V) after transplantation and the strong association with the development of grade 3 PGD. Previous studies have reported higher incidence of any grade of PGD in presence of multiple preexisting antibodies prior to lung transplantation [3,9]. However, we focused particularly on the rapid “de novo” production following lung transplant and only on antibodies against col.(V). Clearly, the sample size is not large enough to determine the risk of developing PGD following rapid seroconversion.

In our proposed mechanism of pathogenicity, ischemia-reperfusion injury after lung transplantation induces remodeling events in the lung interstitium, rapidly exposing col.(V) [6]. The exposure of col.(V) during early post-transplant inflammation leads to loss of self-tolerance and the production of de novo auto-antibodies. This may facilitate the development clinically of significant PGD. Col(V) may act as a danger signal characterized by a rapid cell-surface recognition, inducing specific transcriptional pathways and prompt antibodies production. This

	PGD grade 3 at 72 h n, (%)	No PGD n, (%)	*p* value
Group 1 (n= 134)	58 (43.26%)	76 (56.72%)	>0.05
Group 2 (n= 23)	6 (26.09%)	17 (73.09%)	>0.05
Group 3 (n=13)	5 (38.46%)	8 (61.54%)	>0.05
Group 4 (n=8)	8 (100%)	0(0%)	*0.01*

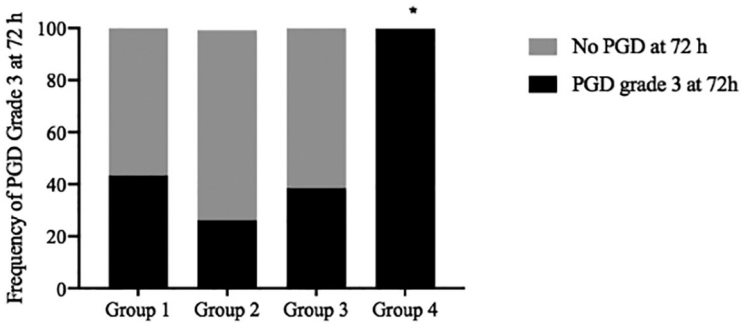

Fig. 4. Development of de novo anti-col(V) Abs is associated with PGD grade 3 after lung transplantation. Frequency of grade 3 PGD at 72 h in 188 lung transplant recipients. PGD grade was determined using the ISHLT consensus definition. Anti-col(V) antibodies (Abs) were analyzed pre-transplant, and post-transplant at 6 h and 24 h. Group 1 had no anti-col(V)Abs pre or post-transplant; Group 2 had persistent positive anti-col(V) Abs pre and post-transplant; Group 3 demonstrated positive anti-col(V) Abs only pre-transplantation; Group 4 developed anti-col (V) within 24 h after transplantation. Kruskal-Wallis H test showed a statistically significant incidence of PGD grade 3 post-transplant (^{2} (3) =10.725, p = .01) after development of anti-col(V) Abs within 24 h after transplantation.

danger response could be associated with the development of PGD in a susceptible group of patients. Our work shows that seroconversion from anti-col(V) negative to anti-col(V) positivity in serum may be a new marker for early identification of PGD, and eventually a new target for future studies to implement possible therapeutic interventions.

Further studies are necessary to confirm the role of col.(V) as danger signal focusing on identifying the cellular receptor, the intracellular signaling pathways that lead to the production of anti-col(V) Abs and further defining the relationship between rapid production of anti-col (V) Abs and PGD.

Authorship contributions

LZ, RJS, RSS: Perform Research, Data analysis, Writing manuscript.
KR, MY, AJF, EAM, MGD, AME: Perform Research.
JDC, JMD, MWG, DSW: Design Study, Data analysis, Writing manuscript.

Declaration of Competing Interest

Dr. Wilkes is Founder and Chief Scientific Officer of ImmuneWorks, Inc. The other authors have no conflicts of interest to disclose.

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Appendix A. Supplementary data

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References

[1] J.M. Diamond, J.C. Lee, S.M. Kawut, et al., Clinical risk factors for primary graft dysfunction after lung transplantation, Am. J. Respir. Crit. Care Med. 187 (5) (2013) 527–534.
[2] R.J. Shah, J.M. Diamond, Primary graft dysfunction (PGD) following lung transplantation, Semin Respir Crit Care Med. 39 (2) (2018) 148–154.
[3] V. Tiriveedhi, B. Gautam, N.J. Sarma, et al., Pre-transplant antibodies to Kalpha1 tubulin and collagen-V in lung transplantation: clinical correlations, J. Heart Lung Transplant. 32 (8) (2013) 807–814.
[4] J.L. Bobadilla, R.B. Love, E. Jankowska-Gan, et al., Th-17, monokines, collagen type V, and primary graft dysfunction in lung transplantation, Am. J. Respir. Crit. Care Med. 177 (6) (2008) 660–668.
[5] R. Fernandez, S. Chiu, K. Raparia, et al., Humoral human lung allograft rejection by tissue-restricted non-HLA antibodies, Ann. Thorac. Surg. 102 (4) (2016) e339–e341.

[6] T. Iwata, A. Philipovskiy, A.J. Fisher, et al., Anti-type V collagen humoral immunity in lung transplant primary graft dysfunction, J. Immunol. 181 (8) (2008) 5738–5747.
[7] L. Milross, R. Hachem, D. Levine, A.R. Glanville, Lung autoantibodies: ready for prime time? J. Heart Lung Transplant. 37 (2) (2017) 179–181 (Feb 2018).
[8] A. Bharat, D. Kreisel, Immunopathogenesis of primary graft dysfunction after lung transplantation, Ann. Thorac. Surg. 105 (3) (2018) 671–674.
[9] A. Bharat, D. Saini, N. Steward, et al., Antibodies to self-antigens predispose to primary lung allograft dysfunction and chronic rejection, Ann. Thorac. Surg. 90 (4) (2010) 1094–1101.
[10] S.A. Yousem, S.R. Suncan, N.P. Ohori, E. Sonmez-Alpan, Architectural remodeling of lung allografts in acute and chronic rejection, Arch. Pathol. Lab. Med. 116 (11) (1992) 1175–1180.
[11] W.J. Burlingham, R.B. Love, E. Jankowska-Gan, et al., IL-17-dependent cellular immunity to collagen type V predisposes to obliterative bronchiolitis in human lung transplants, J. Clin. Invest. 117 (11) (2007) 3498–3506.
[12] T. Iwata, M. Chiyo, S. Yoshida, et al., Lung transplant ischemia reperfusion injury: metalloprotease inhibition down-regulates exposure of type V collagen, growth-related oncogene-induced neutrophil chemotaxis, and tumor necrosis factor-alpha expression, Transplantation. 85 (3) (2008) 417–426.
[13] D.S. Wilkes, T. Chew, K.R. Flaherty, et al., Oral immunotherapy with type V collagen in idiopathic pulmonary fibrosis, Eur. Respir. J. 45 (5) (2015) 1393–1402.
[14] G. Shankar, V. Devanarayan, L. Amaravadi, et al., Recommendations for the validation of immunoassays used for detection of host antibodies against biotechnology products, J. Pharm. Biomed. Anal. 48 (5) (2008) 1267–1281.
[15] T. Fowler, A.S. Garruss, A. Ghosh, et al., Divergence of transcriptional landscape occurs early in B cell activation, Epigenetics Chromatin 8 (2015) 20.
[16] J.D. Christie, N. Robinson, L.B. Ware, et al., Association of protein C and type 1 plasminogen activator inhibitor with primary graft dysfunction, Am. J. Respir. Crit. Care Med. 175 (1) (2007) 69–74.
[17] J.D. Christie, C.V. Shah, S.M. Kawut, et al., Plasma levels of receptor for advanced glycation end products, blood transfusion, and risk of primary graft dysfunction, Am. J. Respir. Crit. Care Med. 180 (10) (2009) 1010–1015.
[18] J.D. Christie, S. Bellamy, L.B. Ware, et al., Construct validity of the definition of primary graft dysfunction after lung transplantation, J. Heart Lung Transplant. 29 (11) (2010) 1231–1239.
[19] J.C. Lee, J.D. Christie, Primary graft dysfunction, Proc. Am. Thorac. Soc. 6 (1) (2009) 39–46.
[20] G.J. Laurent, Lung collagen: more than scaffolding, Thorax. 41 (6) (1986) 418–428.
[21] D. Eyre, Collagen of articular cartilage, Arthritis Res. 4 (1) (2002) 30–35.
[22] S. Jurcevic, M.E. Ainsworth, A. Pomerance, et al., Antivimentin antibodies are an independent predictor of transplant-associated coronary artery disease after cardiac transplantation, Transplantation. 71 (7) (2001) 886–892.
[23] N.E. Holodick, N. Rodriguez-Zhurbenko, A.M. Hernandez, Defining natural antibodies, Front. Immunol. 8 (2017) 872.
[24] M.V. Kuleshov, M.R. Jones, A.D. Rouillard, et al., Enrichr: a comprehensive gene set enrichment analysis web server 2016 update, Nucleic Acids Res. 44 (W1) (2016) W90–W97.
[25] J.A. Blake, J.T. Eppig, J.A. Kadin, J.E. Richardson, C.L. Smith, C.J. Bult, Mouse genome database (MGD)-2017: community knowledge resource for the laboratory mouse, Nucleic Acids Res. 45 (D1) (2017) D723–d729.
[26] R.R. Hachem, V. Tiriveedhi, G.A. Patterson, A. Aloush, E.P. Trulock, T. Mohanakumar, Antibodies to K-alpha 1 tubulin and collagen V are associated with chronic rejection after lung transplantation, Am. J. Transplant. 12 (8) (2012) 2164–2171.
[27] D.S. Wilkes, Autoantibody formation in human and rat studies of chronic rejection and primary graft dysfunction, Semin. Immunol. 24 (2) (2012) 131–135.
[28] B. Almoguera, A. Shaked, B.J. Keating, Transplantation genetics: current status and prospects, Am. J. Transplant. 14 (4) (2014) 764–778.
[29] Y. Suzuki, E. Cantu, J.D. Christie, Primary graft dysfunction, Sem. Respirat. Crit. Care Med. 34 (3) (2013) 305–319.