The Th17 pathway in cystic fibrosis lung disease

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Online Supplementary information

Materials and Methods

Further details of patients

Exclusion criteria for all groups were an abnormal clotting profile or any cardiorespiratory instability during the procedure, as judged by a Consultant paediatric anaesthetist who was independent of the study group. A respiratory exacerbation was defined as an increase in respiratory symptoms severe enough to require admission for intravenous antibiotics. Of the established CF patients, bronchoscopy was performed during respiratory exacerbations in 29 patients. 3 were clinically stable and were undergoing procedures such as portacath insertion. 1 patient could not be easily classified into either group as she had received a course of intravenous antibiotics for a respiratory exacerbation, proceeded to have a portacath inserted at the end of this course and had a bronchoscopy at time of surgery, she was therefore left out of the analysis comparing CF patients undergoing respiratory exacerbations with those who were stable.

A randomized selection of biopsies of 10 patients from each group were used for the double staining experiments. We also performed double staining experiments on samples of explanted lung from 3 adult CF transplant patients.

Table E1. Characteristics of patients who had endobronchial biopsies analysed

	CF established	CF newly diagnosed	Non-CF Bronchiectasis	Controls
Subjects	24	13	14	7
Age Median (Interquartile range) years	9.3(5.3-12)	2(0.3-4)	9(7.5-13.4)	9.5(5.9-10.4)
Male gender No. (%)	8(33%)	5(38%)	2(14%)	1(14%)

	CF established	CF newly diagnosed	Non-CF Bronchiectasis	Controls
FEV1 Median(Interquartile range) % predicted	52(44-61)	N/A	85(75-89)	86(74-107)
No. of patients with respiratory exacerbation at time of bronchoscopy	21	3	14	0

Table E2. Characteristics of patients in whom BALF was analysed

	CF established	CF newly diagnosed	Non-CF bronchiectasis	Controls
Subjects	29	10	17	11
Age Median (Interquartile range) years	9.3 (6.2-12.8)	2.7 (0.5-4.1)	8.9 (7.7-11.9)	5.9 (0.9-10.4)
Male gender No. (%)	9 (31%)	5 (50%)	4 (24%)	4 (36%)
FEV1 Median(Interquartile range) % predicted	52 (45-72)	N/A	81 (72-88)	98 (74-111)
No. of patients who could do lung function	25	0	17	5
No. of patients with respiratory exacerbation at time of bronchoscopy	25	3	17	0

Protocol for flexible bronchoscopy

All bronchoscopies were performed under general anaesthesia. Olympus BF-XP40 (2.8 mm external diameter), BF-3C20 or 3C40 (3.6 mm), BF-MP60 (4.0 mm videobronchoscope) or BF-P20D (4.9 mm) bronchoscopes (KeyMed, Southend-on-Sea, Essex, UK) were used as appropriate to the size of the child. Bronchoalveolar lavage was performed using 3 aliquots of 1ml/kg 0.9% sterile saline (to a maximum of 40mls per aliquot) instilled into the right middle lobe or an area of radiographically-defined abnormality and the returns pooled. Small re-usable forceps (FB-56D, KeyMed) were used with the 2.8 and 3.6mm bronchoscopes and the larger single use forceps (FB-231D, KeyMed) were used with the 4.0 and 4.9mm bronchoscopes. Up to 5 biopsies were taken under direct vision from the subsegmental bronchi of the right lower lobe.

Processing of BALF

An aliquot of the BALF sample was sent to the clinical microbiology laboratory for microscopy, bacteria culture and viral immunofluorescence and culture. An aliquot was also sent to the histology laboratory for differential cell counts. The remainder was placed immediately on ice and processed within 4 hours. Samples were centrifuged (3460 rpm for 10min) and the supernatant frozen at -80˚C for future analysis. The cell pellet was resuspended in RPMI-1640 medium(Sigma) with 10% fetal calf serum to give a concentration of $5x10^5$ cells/ml. Cytospins were performed using 100µl of this cell suspension for each slide at a speed of 500rpm for 4min. After the slides were airdried, they were fixed in methanol for 5min. Immunohistochemistry, double staining for IL-17 and Neutrophil elastase was then performed on the slides as described below.

IL-17 IHC protocol

After dewaxing with Histoclear and rehydrating with alcohol, antigen retrieval was performed by microwaving for 15min in 10mM sodium citrate. Endogenous peroxidase was removed by incubating with hydrogen peroxidase for 30min at room temperature. The Avidin/Biotin Blocking Kit (Vector laboratories SP 2001) was then used (the section was incubated with Avidin D for 15min, washed, then incubated with Biotin for 15min) to remove potential non-specific binding from endogenous biotin or biotin binding proteins, lectins or nonspecific binding substances present in the section. Dako Protein Block (X0909) was applied for 30min at room temperature. The primary antibody (goat Anti-Human IL-17 antibody (R&D systems AF-317-NA)) was added at a dilution of 1:50 and the samples incubated at 4˚C overnight. Biotinylated polyclonal rabbit anti-goat secondary antibody was applied (DAKO, E0466) at a concentration of 1:250 for 1 hour. The Vectastain Elite ABC system (Vector laboratories) was used to localise IL-17 positive cells before counter staining with haematoxylin. A negative isotype control was performed for each sample using goat immunoglobulin at the same concentration as the primary IL-17 antibody.

Double staining IF protocol

Antigen retrieval was by microwaving in Tris EDTA buffer (pH 9) for 15min. Dako Protein Block (X0909) was applied for 30min before the IL-17 antibody (R&D AF-317-NA) was added at a dilution of 1:50 and the samples were incubated at 4˚C overnight. The secondary antibody, AlexaFluor 680 donkey anti goat IgG (Invitrogen A-21084) was applied at a dilution of 1:250 and incubated for 1 hour at room temperature. Following another blocking step, the second primary antibody, anti-CD4 (same as above) was applied and the samples incubated for 1 hour at room temperature. The samples were then incubated in the second secondary antibody AlexaFluor 488 F(ab) fragment of rabbit

anti-mouse IgG (Invitrogen A-21204) at room temperature for 1 hour. ProLong Gold antifade reagent with DAPI (Invitrogen, P36934) was subsequently applied.

Double staining for IL-17 and other antibodies was with a similar protocol as described above with variations as listed in the Table 2. For double staining of γδTCR with IL-17 and Vα24 with IL-17, an additional amplification step was performed where the second secondary antibody used was biotinylated anti-mouse IgG (BA2001, Vectorlabs). Samples were then incubated with Streptavidin Alexa Fluor 488 conjugate (S11223, Invitrogen) for 20min before mounting with ProLong Gold antifade reagent with DAPI.

For each double staining experiment, a negative isotype control was performed as well as 2 further controls, each with just 1 of the primary antibodies and the respective isotype control of the other primary antibody. For γδ T cells, tonsillar tissue was used as a positive control. For NKT cells, splenic tissue was used as a positive control.

Table E3. Details of antibodies used and their respective antigen retrieval methods

Second Primary Antibody	Antigen retrieval	Dilution
CD8 (Monoclonal mouse anti human) Dako, M7103	Microwave 15min in Tris EDTA buffer pH 9.0	1:50
CD68 (Monoclonal mouse anti human) Dako, M0876	Microwave 15min in Tris EDTA buffer pH 9.0	1:100
Neutrophil elastase (Monoclonal mouse anti human) Dako, M0752	Proteinase K (20µg/ml, Sigma Aldrich), at 37C for 10min	1:100

Second Primary Antibody	Antigen retrieval	Dilution
γδTCR[41] (Monoclonal mouse anti human TCR γ chain constant region) Thermo scientific TCR1153, clone γ3.20	Microwave 15min in EDTA buffer pH 8.0	1:20
NKT cells (Monoclonal mouse anti human TCR Vα24) Beckman Coulter IM1588	Proteinase K (20μg/ml, Sigma Aldrich), at 37C for 10min	1:50

Analysis of endobronchial biopsies

Cell counts were performed by a single observer, blinded to the diagnosis. To ensure consistency, only biopses with minimal crush artefact and good morphology, with the presence of epithelium, reticular basement membrane and subepithelial tissue were analysed. The area occupied by muscle, cartilage and glandular tissue was excluded. When a patient had more than one evaluable biopsy, the sum of the number of positive cells in the submucosa was counted and the sum of the submucosal area measured in all the biopsies was used to calculate the number of cells/ mm^2. The sample was excluded when the sum of the total submucosal area was less than $0.1mm^2$. This explains the disparity between the number of patients for which BALF data is available and the number of patients where biopsy data is available. Intraobserver variability was calculated by counting 10 slides on 3 separate occasions. The coefficient of variation was 3.8% for CD8 counts, 5.2% for IL-17+ counts and 7.5% for the CD4 counts.

Luminex Bio-plex Cytokine Assay

The Human 17 plex cytokine assay (Bio-rad) was used to perform cytokine measurements on BALF supernatant as per manufacturer's instructions. BSA (bovine serum albumin) (Sigma-Aldrich) was added as a carrier protein at a concentration of 1%. 50µl of multiplex bead working solution was added to each well of the supplied 96 well filter plate which had been prewet. They were washed twice with Bio-plex wash buffer, the buffer was removed by vacuum filtration. 50µl of sample (or diluted standard for the standard curve) was added to the wells. The filter plate was sealed and covered with aluminium foil, placed on a microplate shaker and incubated for 30min at room temperature. The wells were washed 3 times with Bio-plex wash buffer. 25µl of Bio-plex detection antibody working solution was added to each well and incubated for 30min. The wells were washed 3 times with Bio-plex wash buffer. 50µl streptavidin-PE (Phycoerythrin) was added to each well and incubated for 10min. The wells were washed 3 times. The beads in each well were resuspended with 125µl of Bio-plex assay buffer and the plate was read with the Luminex 100 system. All samples were tested in duplicate. The standards were reconstituted with 0.9% saline with 1% BSA.

Table E4. Concentration of standard curves (pg/ml) for each cytokine tested in Luminex bio-plex cytokine assay.

	Standard 1(pg/ml)	Standard 2 (pg/ml)	Standard 3 (pg/ml)	Standard 4 (pg/ml)	Standard 5 (pg/ml)	Standard 6 (pg/ml)	Standard 7 (pg/ml)	Standard 8 (pg/ml)
IL-1β	11038	2759	690	172.5	43.1	10.8	2.7	0
IL-2	4625	1156	289	72.3	18.1	4.5	1.1	0
IL-4	915	229	57	14.3	3.6	0.9	0.2	0
IL-5	11480	2870	717	179.4	44.8	11.2	2.8	0
IL-6	9441	2360	590	147.5	36.9	9.2	2.3	0
IL-7	5764	1441	360	90.1	22.5	5.6	1.4	0
IL-8	5751	1438	359	89.9	22.5	5.6	1.4	0
IL-10	9126	2281	570	142.6	35.6	8.9	2.2	0
IL-12	10217	2554	639	159.6	39.9	10.0	2.5	0
IL-13	8466	2117	529	132.3	33.1	8.3	2.1	0
IL-17	6915	1729	432	108.1	27.0	6.8	1.7	0
IFNγ	19399	4850	1212	303.1	75.8	18.9	4.7	0

TNFα	21085	5271	1318	329.4	82.4	20.6	5.1	0
MCP1	7209	1802	451	112.6	28.2	7.0	1.8	0
MIP1β	5619	1405	351	87.8	21.9	5.5	1.4	0
GCSF	7755	1939	485	121.2	30.3	7.6	1.9	0
GMCSF	3818	954	239	59.7	14.9	3.7	0.9	0

IL-23 ELISA

The human IL-23 Quantikine immunoassay kit, D2300B, from R&D systems was used as per manufacturer's instructions. 100µl of Assay diluent RD1-22 (a buffered protein solution) was added to each well of the 96 well polystyrene microplate which is coated with a goat polyclonal antibody against the human IL-23 p19 subunit. 100µl of standard, or sample was added to each well. It was then incubated for 2 hours at room temperature on a microplate shaker. The plate was washed 4 times, 200µl of IL-23 conjugate (goat polyclonal antibody against the human IL-23 p40 subunit conjugated to horseradish peroxidase) was added to each well. It was further incubated for 2 hours at room temperature on the shaker. The wells are washed 4 times again. Colour reagent A (Hydrogen peroxide) was mixed with Colour reagent B (tetramethylbenzidine) and 200µl of the resulting solution was added to each well. It was incubated for 30min at room temperature, protected from light. 50µl of Stop solution (2 N sulfuric acid) was added to each well and the optical density read with a microplate reader set to 450nm, with 540nm wavelength correction. Samples were tested in duplicate. The standards used were 2500pg/ml, 1250pg/ml, 625pg/ml, 313pg/ml, 156pg/ml, 78.1pg/ml, 39pg/ml and 0pg/ml. The mean minimal detectable dose quoted by the manufacturers was 6.8pg/ml.

Results

Clinical details of the outlier with raised IL-17 levels in the control patients

Bronchoscopy showed laryngomalacia with arytenoids prolapsing into the laryngeal inlet and tracheobronchomalacia. There were some increased secretions in the lower airways. BALF culture showed no significant bacterial growth, viral immunofluorescence was negative, cytology showed normal numbers of lymphocytes and neutrophils. Besides IL-17 levels which were raised, she also had raised levels of GMCSF, GCSF, IL-1β, IL-6, IL-8, IFNγ, MIP1β, TNF and total cell count in her BALF. Her biopsy histology showed mild basement membrane thickening and mild chronic inflammatory cell infiltrate within the underlying stroma with no eosinophils identified.

Table E5. Cytokines in BALF which had a similar pattern as IL-17 levels in BALF where CF newly diagnosed patients had low levels of cytokines similar to controls, whereas patients with established CF had significantly higher cytokine levels, similar to patients with non-CF bronchiectasis

Cytokine	KW *p value*
GMCSF	0.001
IFNγ	0.001
IL-1β	0.049
IL-8	0.001
MCP1	0.035
IL-4	0.004
TNFα	0.011

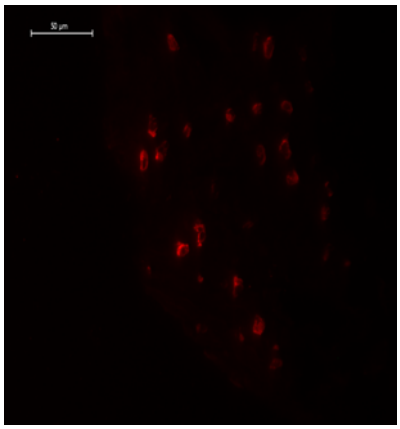
Table E6. Correlations of IL-17 BALF levels with other BALF cytokine levels which were significant but weak.

Cytokine	***p* value**	**Spearman's Correlation Coefficient (R =)**
IL-1β	<0.001	0.71
GMCSF	<0.001	0.5
IL-2	<0.001	0.54
IFNγ	<0.001	0.65
TNF	<0.001	0.67
IL-8	<0.001	0.52
MCP1	<0.01	0.34
MIP1β	<0.05	0.27

Figure E1a.IL-17+ cells staining red 1b.Neutrophil elastase+ cells staining green

1c.Double staining for IL-17 and Neutrophil elastase in CF paediatric endobronchial biopsy

1d. Negative control



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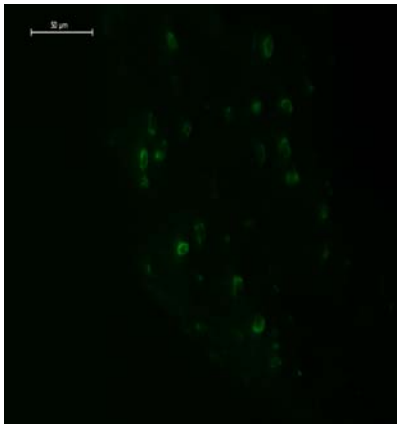

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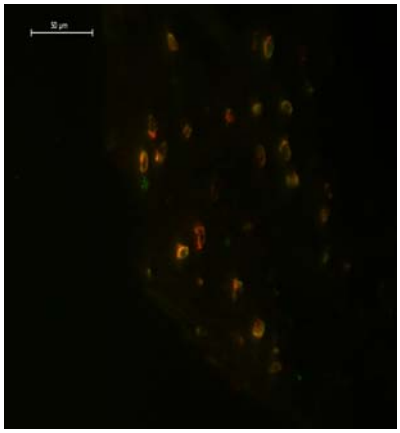

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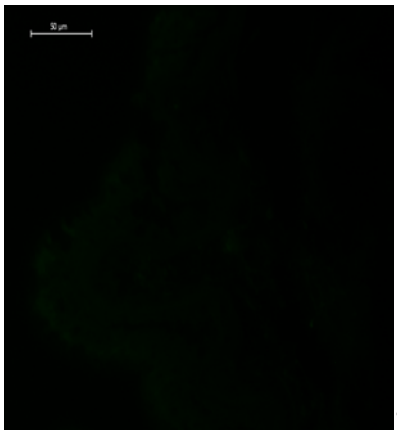
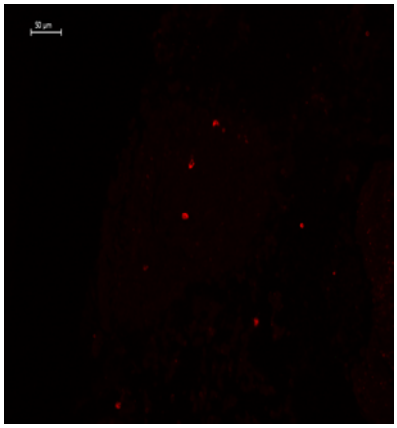

1d

Figure E2a. IL-17+ cells staining red 2b.γδTCR+ cells staining green 2c. Double staining for IL-17 and γδTCR in CF paediatric endobronchial biopsy 2d. Negative control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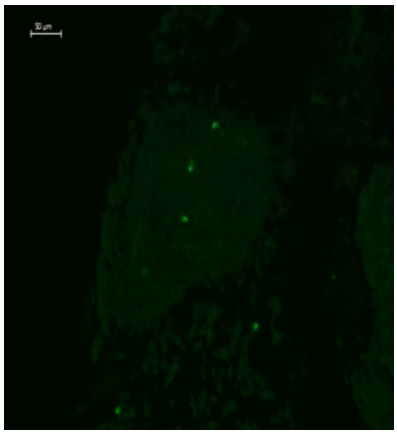
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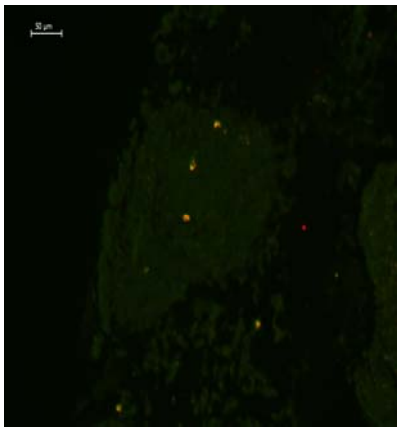
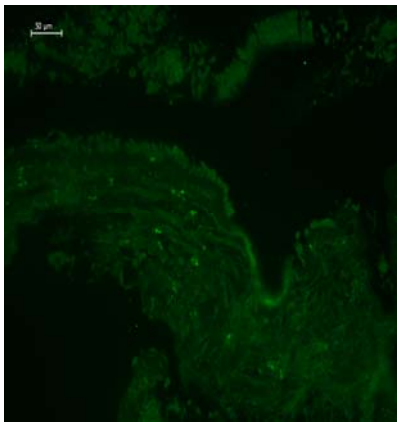


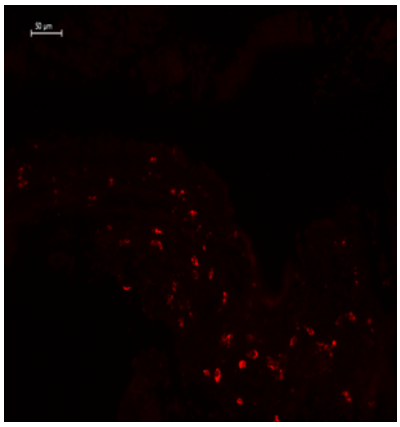
2c

2d

Figure E3a.IL-17+ cells staining red 3b.NKT cells staining green 3c.Double staining for IL-17 and the NKT cell marker Vα24 TCR in CF adult explanted lung tissue 3d. Negative control



3a



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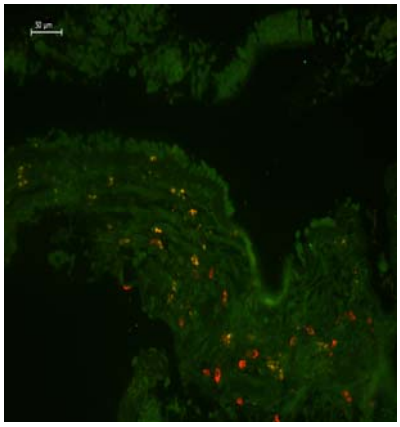

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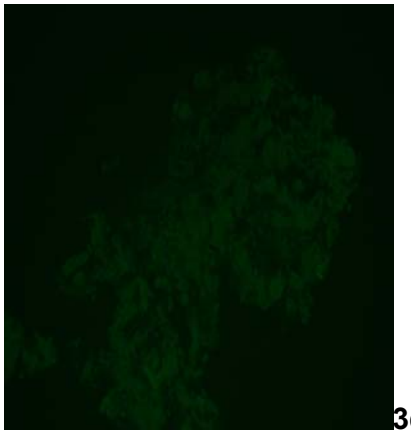
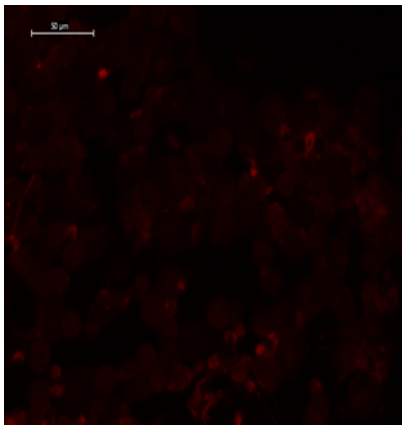


3d

Figure E4. Immunofluorescence of BALF cytospins from paediatric CF patients 4a. IL17+ cells staining red 4b.Neutrophil elastase+ cells staining green 4c. Double staining for IL17 and Neutrophil elastase 4d. Negative control (DAPI)



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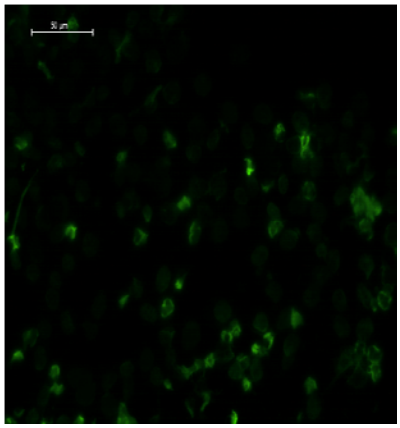

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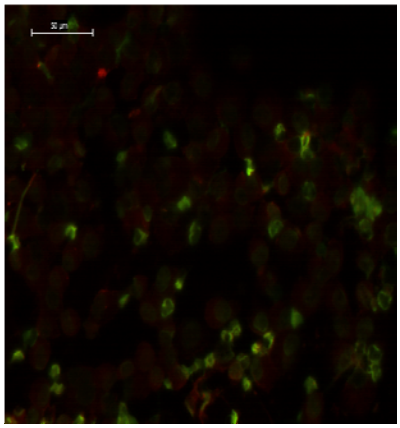

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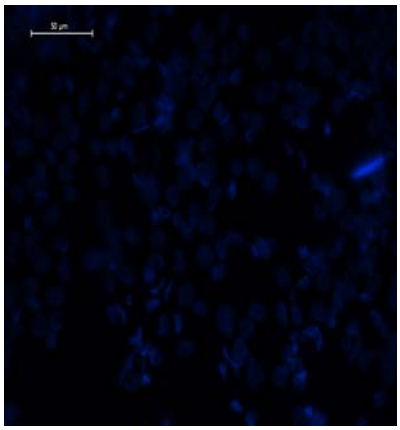

4d