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Abstract

Purpose: To evaluate the presence of interleukin-17 (IL-17)-producing cells in patients with geographic atrophy (GA).

Methods: In this short report, we analyzed IL-17, CD3 and IBA-1 expression by immunohistochemistry on paraffin-embedded sections from 13 donors with a known history of GA, confirmed by fundus appearance and histology, and 7 age-matched control donors.

Results and conclusion: we show that IL-17+ cells are found near areas of retinal pigmented epithelium (RPE) atrophy in eyes of GA patients. IL-17^{+} cells mainly localized to CD3^{+}cells, which identifies T lymphocytes, as well as IBA-1^{+} cells, which identifies mononuclear phagocytes. Therefore IL-17 could be involved in the pathological mechanisms that contribute to degeneration observed in GA.

Keywords

Interleulin-17, lymphocytes, CD3, choroid, geographic atrophy, age-related macular degeneration

Introduction

Age-related Macular Degeneration (AMD) is a leading cause of irreversible blindness in elderly people in the industrialized world (1). There are two late forms of AMD: the exudative or wet form defined by choroidal neovascularization (CNV) and the slow developing atrophic form known as Geographic Atrophy (GA). There is no efficient therapy for GA at the moment. GA is characterized by choroidalthinning (2)and a slowly expanding lesion of photoreceptor andretinal pigment epithelium (RPE) degeneration and dysfunction (3). GA is a complex multifactorial event influenced by: aging (4), environmental factors such as smoking history (5), oxidative stress (6, 7) and geneticpredisposition, particularly haplotypes containing polymorphisms of Complement Factor H (CFH)(8) andARMS2/HTRA1 (9). Moreover, low grade inflammation (10, 11)and accumulation of mononuclear phagocytes in the subretinal space areclearly associated with GA(12, 13, 14).Adaptive immunity probably also play a role in AMD pathophysiology (15). Indeed, 30 years ago, Penfold *et al.* detected the presence of lymphocytes in the eyes of GA and exudative AMD patients and they proposed that they may play a role in RPE atrophy and breakdown of Bruch's membrane (16, 17). Moreover $CD8^+$ cells have been observed in the choroid of frozen sections of eyes from advanced AMD patients with or without drusen or fibrovascular scar (18). Interestingly, Gregerson *et al.*(19) showed that cytotoxic $CD8^+$lymphocytes could kill murine RPE cells*in vitro* by induction of apoptosis in an antigen-dependent manner. Faber *et al.* (20)demonstrated that the presence of $CD56^+CD28^-$ memory T cells in the blood of AMD patients is associated with a3.5 foldincrease of the risk of developing AMD. The risk of AMD is increased up to 13.3 times in the case of persons with enhanced levels of memory T cells in blood and exhibiting at least one CFH H402Y risk allele.

Interleukin-17 A (IL-17) is a cytokinethat contributes to the pathogenesis of inflammatory and auto-immune diseases(21, 22) but its role is not restricted to these pathologies(23). A major source of IL-17 is a lineage of T cells known as $CD4^{+}$T helper 17 cells (Th17 cells) which differ from the Th1 and Th2 subsets (24). IL-17 is secreted by other cell types of innate and adaptive immune systemsnotably macrophages(25, 26). In GA, an increase of IL-17 immuno-positivity has been reported in GA eyes compared to control subjects, but the IL-17-producing cells could not be identified(27). Furthermore, genetic variants of IL-17 have been shown to be associated with AMD(28), while the reported association of hypo-methylation of the IL-17 receptor(27) was not reproducible in another study(29).

In this short report, we show that $IL\text{-}17^{+}$ cells are not found in un-affected donor eyes, but accumulate in the choroid of GA donors near areas of RPE atrophy. They mainly co-localize with CD3 staining, which identifies Tlymphocytes, and IBA-1 staining that identifies cells of the monocyte/macrophage family. IL-17, released from infiltrating immune cells, could play an important role in GA pathogenesis.

Materials and Methods

Donor Eyes

Donor eyes with or without a known history of AMD, with GA lesions visible on postmortem fundus photographs confirmed by histological evaluation and controls, were obtained from theMinnesota Lions Eye bank (7 control maculae from 7 patients (5 men and 2 women), meanage 84+/- 2.6 years; 13 GA donor maculae from 11 different patients (4 men and 7 women),mean age 83.7 +/- 2.4 years). Fundus photographs were taken with using a macroscope andadapted camera by the Minnesota Lions Eye bank. Donor eyes without a

known history of eye diseases and no signs of RPE or retinal lesions were considered as being control eyes. Informed consent was obtained for alldonor eyes from Minnesota Eye bank and experiments conformed to the principles set out inthe WMA Declaration of Helsinki.

Histology and Immunohistochemistry

The posterior segment was fixed 4h in 4% paraformaldehyde. 8µm horizontal sections of paraffin embedded human tissues crossing the optic nerve and perifovea were cut with amicrotome (Microm Microtech France). The sections were de-paraffinized, by incubation inSafesolv and rehydration was performed in serial baths of alcohol/water. Some sections ofeach sample were stained with toluidin blue for histology. Antigen retrieval was performed inboiling EDTA buffer for 15 minutes. Following inhibition of endogenous peroxidases for 20 minutes in 0.3% H2O2 (Sigma H1009, 1:100 in PBS), sections were blocked with 1% purified horse or goat serum and then exposed overnight to rat anti-human CD3e (MCA1477, Serotec), goat anti-human IL-17 (AF-317-NA, R&D systems) or rabbit anti-IBA-1 (Wako). Alexa-fluorescent-conjugated secondary antibody (1:400; Life Technologies) was used to reveal primary antibodies. Sections were counterstained with 4-6-diamino-2-phenylindole (DAPI). They were imaged with a DM5500 microscope (Leica) or a confocal microscope (LSM 710, Zeiss). Alternatively, anti-IL-17 primary antibodies were incubated with peroxidase labeled horse anti-goat IgG (PI-9500) (Vector laboratories) and revealed with peroxidase substrate kit (SK-4600, Vector laboratories). IL-17+ cells were counted in the choroid and retina of central control sections and central section's containing GA lesions and divided by the length of the section for control retina and the length of the GA lesion for AMD eyes (cells/mm). The quantifications were performed on substrate IL17-stained sections. Additionally we performed double labeling of IL-17 and CD3e on all GA sections and IL-17 and IBA-1 staining on 3 sections. Staining that omitted the primary antibodyserved

as negative controls. Final image processing was performed using Adobe Photoshop(Mountain View). All images are representative of at least 3 stainings performed with similarresults.

Statistical analysis

Graph Pad Prism 6 (GraphPad Software) was used for data analysis and graphic representation. We analyzed 7 control maculae from 7 patients and 13 GA donor maculae from 11 patients. All values are reported as means ± SD. Statistical comparisons used non–parametric Mann-Whitney test. *P*-values less than 0.05 were considered statistically significant.

Results and Discussion

Healthy donor eyes revealed a visible fovea on post-mortem funduscopy and a regularhistology of the parafovea, recognizable by multilayered ganglion cell nuclei (Fig. 1A and B).

GA lesions were visible on post-mortem fundus pictures (Fig. 1C) and confirmed histologically by the missing RPE and thinned photoreceptor cell layer in the lesion of the parafovea (multilayered ganglion cell layer) in the absence of vessels penetrating Bruchs membrane (as observed in neovascular AMD) or a fibroglial scar (Fig. 1D).

InGA sectionscontaining the atrophic lesions (illustrating fundus and histological section in Figure 1, compared to normal donor sections), we identified IL-17^{+}cells by immunohistochemistry. Numerous IL-17^{+}cells were identified in all 13 GA sections, but never in control donor sections (Fig.2A). IL-17^{+}cells were mainly located within the choroid in proximity to the RPE lesion (Fig. 2B to E) and very rarely found in the adjacent retinaor (Fig.2C and G). Quantification of IL-17^{+} cells per millimeter of section show a significant

increase of IL-17^{+}cells in GA sections compared to control donor sections (Fig.2F) and that they are mainly located in the choroid. (Fig. 2G).

IL-17 can be secreted by different cell types, but notably by T lymphocytes and macrophages (25). Indeed the round nucleus and small cytoplasm of numerous IL-17^{+} cells (Fig. 2E) suggested that T lymphocytes participate in IL-17 production in GA. Confocal microscopy of double immuno-labelling of IL-17 and CD3 (specifically expressed by T cells (30)) confirmed that an important portion part of IL-17-producing cells (Fig. 3B) are CD3^{+} T lymphocytes (Fig. 3A, merge 3C). Furthermore, double labeling using the anti-IL17 antibody (Fig. 3E and Fig. 3H) and an anti-IBA-1 antibody (monocyte/macrophage marker, Fig. 3D and G, merge 3F and I) revealed that a sub-population of macrophages participate in IL-17 expression in GA. In our experiments, approximately 50% of the infiltrating IL-17^{+} cells corresponded to CD3^{+} T cells. IBA-1^{+}IL-17^{+} macrophages were less frequentthan IL-17^{+} CD3^{+} T cells and represented only a small fraction of IBA-1^{+} macrophages (Fig. 3D to F). Nevertheless, future studies are necessary to identify the remaining IL-17 expressing cell types and to elucidate the exact proportions of all the different IL-17-expressing cell populations in GA eyes.

In conclusion, our results indicate that IL-17+ cells are invariably present in GA lesions but not observed in aged-matched control sections confirming earlier studies of increased IL-17 staining in GA (31, 32). Our study identifies T lymphocytes as an important source of IL-17 in GA. We also show that IBA-1^{+} monocytes/macrophages participate in the IL-17 production in the choroid of GA patients. This local IL-17 production could contribute to the increased IL-17 serum concentration found in AMD patients compared to aged matched controls (33). Moreover our observations suggest that T cells and monocytes/macrophages could be responsible for the significant elevation of IL-17 mRNA in maculae of eyes from AMD

patients compared to control eyes (34). It is yet unclear if and how IL-17 contributes to the GA pathological processes. IL-17 has been shown to directly deteriorate RPE in vitro (32, 35). Moreover IL-17 production has been associated with retinal and RPE lesions in the Nrf2-/- mice model of AMD (36) in vivo and IL-17 could contribute to RPE degeneration in GA.

Figure Legends

Figure 1: Post-mortem fundus picture and histology of donor eyes.

Fundus photographs and toluidin blue stained section of a representative control donor (A and B) and a GA patient (C and D). Scale bar: 50 μm. RPE: retinal pigment epithelium; AZ: atrophic zone.

Figure 2: IL-17$^+$ cells accumulate in eyes of GA patients.

A: IL-17 immuno-staining in the choroid of eye from control donor without degeneration. B and C-E: IL-17 immuno-staining in the retina and choroid of eyes from 2 different GA patients. D: close-up of immuno-staining in C. E: close-up of immuno-staining in D. F: Quantification of IL-17$^+$ cells per mm2 of section in eyes of control donors and GA patients (n=7 and 13 respectively). G: Distribution of IL-17-producing cells per mm^2 of section in eyes of GA patients (n=13). Ctrl: eyes from control donor patients without GA; RPE: retinal pigmented epithelium. Arrows indicate red stained IL-17$^+$ cells. All values are represented as mean ± SD. Scale bar: 20 μm in all images.

Figure 3: CD3$^+$ T cells and IBA-1$^+$ macrophages constitute major IL-17-producing cell types in GA.

A-C: double immuno-staining for IL-17 (A) and CD3e (B, merged image C) in choroid of a

GA patient. D-F: Low power double immuno-staining for IL-17 (D) and IBA-1 (E, merged image F) in choroid of a GA patient.A sub-population of IBA-1^+ macrophages express IL-17 while several others do not. G-I: Close up of immuno-staining in D-F respectively showing a single IBA-1^+ macrophage expressing IL-17. Arrows indicate double stained cells and stars indicate autofluorescence. Immuno-staining omitting the primary antibodies served as controls; double labeling procedures were performed on at least 3 different samples. Scale bar: 20 μm in all images. RPE: retinal pigmented epithelium.

Author contributions

S.C., W.R. and F.S. designed the study, performed experiments, analyzed data and wrote the paper; S.Land X.G. performed experimentsor analyzed data.

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