IL-6 and IL-8 synthesis in tumor cells in our assay. To prove this assumption, MC supernatants were pre-incubated with a neutralising antibody against TNF-α or isotype control antibody before stimulation of the cancer cells. When TNF-α was neutralised, MC–mediated augmentation of IL-8 mRNA expression was almost completely lost and IL-8 concentration decreased to basal levels in Mel-1, SCL-1, SCC-12 and SCC-13 cells (Fig. 2a–d grey bars). Regarding IL-6 mRNA expression, blockade of TNF-α in MC supernatants also abrogated the increase in expression in SCL-1 and SCC-12 cells, but led to an only 50 percent inhibition in SCC-13 cells (Fig. 1d–f). Thus, regulation of IL-6 in SCC-13 cells seems not only processed through TNF-α but probably also through other factors like histamine or tryptase. Similar to our results obtained in SCC-13 cells, TNF-α seemed not responsible for IL-8 synthesis in Mel-4 cells. (Fig. 2e). However, stimulation of Mel-4 cells with histamine, another MC mediator, led to increased gene expression of IL-8 (Fig. 2f). Inhibition experiments with H1- or H2-receptor antagonists desloratadine or famotidine confirmed that up-regulation of IL-8 synthesis in Mel-4 cells was histamine dependent and mediated via the H2 receptor (Fig. 2f).

## Conclusions

Using a novel model to study the communication between human MCs and human tumor cells derived from the same tissue site, we have been able to show that normal human MCs communicate with tumor cells and that TNF-α and histamine are key players in this process. Surprisingly, melanoma and squamous carcinoma cells differed in their response to MC-derived TNF-α: while IL-8 expression and release were up-regulated in melanoma and SCC cells, IL-6 was unaltered in melanoma cell lines, but up-regulated in SCC cell lines. In the SCC lines, MCs further increased constitutive IL-6 and IL-8 mRNA generation, whereas in the melanoma cell lines, Mel-1, Mel-2 and Mel-4, which exhibit an only marginal constitutive IL-8 production, IL-8 synthesis was enhanced by MC-derived TNF-α and/or histamine, far exceeding baseline levels.

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## Conflict of interest

The authors have no conflicting interest.

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## Supporting Information

Additional Supporting Information may be found in the online version of this article:

**Figure S1.** Determination of TNF-α protein levels in co-culture supernatants.

**Figure S2.** Induction of IL-6 and IL-8 mRNA expression in mast cell/cancer cell co-cultures.

**Appendix S1.** Material and Methods.

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Letter to the Editor

# Lesional Th17 cells and regulatory T cells in bullous pemphigoid

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**Abstract:** Th17 cells play crucial roles in the pathogenesis of autoimmune diseases. We previously reported that Th17 cells are recruited to the lesional skin in pemphigus vulgaris (PV) and pemphigus foliaceus (PF). The aim of this study was to evaluate


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lesional Th17 cells and Treg cells in bullous pemphigoid (BP). Correlations between these cells and disease severity of BP were also evaluated. Immunohistochemical studies showed that both IL-17+ and Foxp3+ cells were present in higher numbers in BP lesions, compared with control skin. IL-17/CD4 ratio in BP was significantly higher than that in PF. Foxp3/CD4 ratio in BP was significantly less than that in either PV or PF. There were no obvious correlations between these cells and disease severity of BP. This study suggests that, compared with pemphigus, BP shows more Th17 cell-related inflammation and less Treg-related regulation.

**Key words:** bullous pemphigoid – IL-17 – pemphigus foliaceus – pemphigus vulgaris – Th17 – Treg

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## Background

Th17 cells, characterized by interleukin-17 (IL-17) production, play crucial roles in the pathogenesis of autoimmune diseases (1). We previously reported that Th17 cells are recruited to the lesional skin in pemphigus vulgaris (PV) and pemphigus foliaceus (PF) (2).

## Questions addressed

The aim of the present study was to evaluate the status of lesional Th17 cells and regulatory T cells (Treg) in bullous pemphigoid (BP) by an immunohistochemical approach.

## Experimental design

Twenty-five patients (15 men and 10 women, 32–92 years old, average 71.84 years) were allocated to this study. The data on PV and PF were retrieved from our previous study (2). All patients were diagnosed by histological subepidermal blister, linear IgG deposition at the basement membrane zone and detection of circulating autoantibodies to BP antigens by an enzyme-linked immunosorbent assay (ELISA). The severity indexes in the 24 BP cases (unknown in one case), ranged from 2 to 10 (average: 5.875); they were scored according to the diagnostic criteria of the Specified Disease Treatment Research Program by the Japanese Ministry of Health, Labour and Welfare.

The lesional skin biopsy specimens were obtained before treatment. The control skin was taken from three healthy people. One half of each specimen was used for histological analyses. The rest of the specimens were used for immunohistochemical studies; goat anti-human IL-17 antibody (R&D, Minneapolis, MN, USA), mouse anti-human Foxp3 (Abcam, Cambridge, UK) and mouse anti-human CD4 antibodies (Nichirei, Tokyo, Japan) were used as primary antibodies. The counts of IL-17+, possibly Th17 cells (IL-17); Foxp3+, possibly Treg cells (Foxp3) and CD4+ cells (CD4) in two sections were evaluated. The ratio of IL-17+ or Foxp3+ count to CD4+ count (IL17/CD4, Foxp3/CD4, respectively) was also evaluated. IL-17 levels in BP patient sera and control sera were evaluated by ELISA.

We analysed correlations between two groups of data as follows: (a) IL-17/CD4 vs CD4, (b) Foxp3/CD4 vs CD4, (c) IL-17/CD4 vs age at onset (d) Foxp3/CD4 vs age of onset, (e) IL-17/CD4 vs maximal dose of prednisolone (PSLmax: as the index for disease severity), (f) Foxp3/CD4 vs PSLmax, (g) IL-17/CD4 vs BP180 ELISA index (BP180), (h) Foxp3/CD4 vs BP180, (i) IL-17/CD4 vs severity index, (j) Foxp3/CD4 vs severity index, (k) number of eosinophils (Eos) vs IL-17/CD4, (l) Eos vs Foxp3/CD4 and (m) Eos vs severity index.

## Results

Both IL-17+ and Foxp3+ cells were present in higher numbers in BP lesions (Fig. 1a–c), compared to control skin (Fig. 1d–f). The IL-17/CD4 ratio in BP was 16.28%, significantly higher than that in PF (1.8%) and not significantly but relatively higher than that in PV (5.2%) (Fig. 1g). The Foxp3/CD4 ratio was 5.52%, which was significantly lower than that in either PV or PF (more than 20%) (Fig. 1h). The Th17/Foxp3 ratio in BP was significantly higher (2.95%) than those in PV and PF (Fig. 1i). The serum IL-17, assessed by ELISA, was significantly higher (3.6-fold) in BP

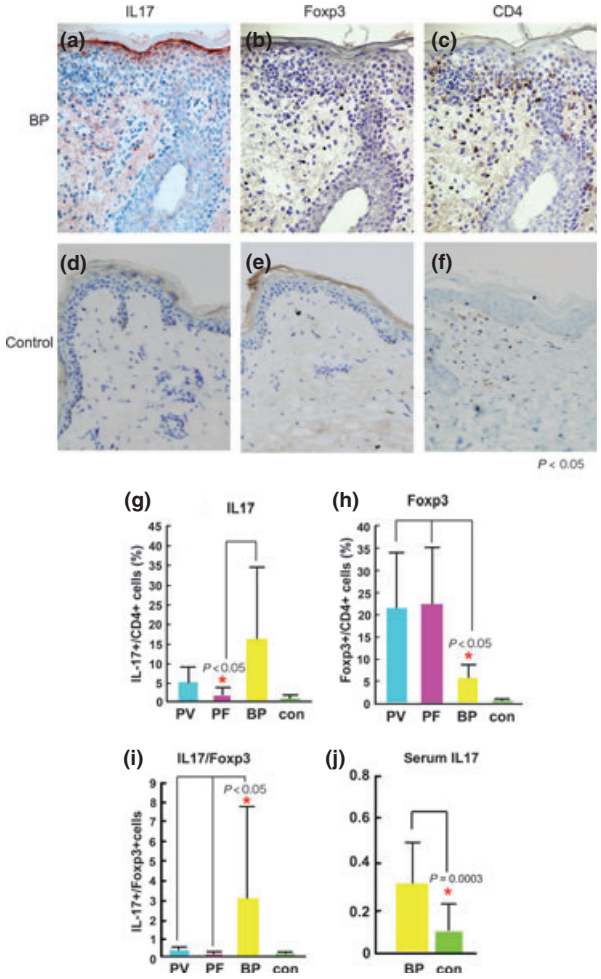


**Figure 1.** Immunohistochemical studies of lesional skin specimens (a–c) and control skin specimens (d–f). Positively stained lymphocytes were detected by antibodies to IL-17 (a, d), Foxp3 (b, e) and CD4 (c, f). Quantification of the number of IL-17-producing (g), Foxp3-expressing (h) and the ratios of IL-17-producing cells/Foxp3 expressing cells (i) in BP, PV, PF and normal control (con). The significance of the differences was assessed by an unpaired *t*-test. (j) Serum IL-17 levels in BP and normal control. BP, bullous pemphigoid; PV, pemphigus vulgaris; PF, pemphigus foliaceus.

serum than in control serum (Fig. 1j). Although not statistically significant as assessed by Spearman's rank correlation coefficient ($P > 0.05$), some pairs of parameters showed tendencies to correlate, i.e., negative correlations are seen in [IL-17/CD4 vs CD4] (Figure S1a), in [Foxp3/CD4 vs CD4] (Figure S1b) and in [Foxp3/CD4 vs BP180] (Figure S1h). Other pairs showed no correlation (Figure S1c–g, i–m).

## Conclusions

This is the first study in which lesional IL-17+ cells and Foxp3+ cells have been quantitatively evaluated in BP. The numbers of Th17 cells were increased in BP and PV skin lesions, but not in PF lesions, while the number of Treg cells in BP lesional skin was significantly smaller than those in PV and PF lesional skin.

The first issue that should be considered is that the numbers of IL-17+ cells were increased in BP and PV, but not in PF. A potential role of Th17 cells in BP was recently suggested (1), because an increased recruitment of IL-17+ cells in the lesional tissue was observed in mucous membrane pemphigoid (MMP), another pemphigoid member (3). Although the role of Th17 cells in the pathogenesis of autoimmune diseases is unresolved, they may be the initiators of diseases (2). Alternatively, Th17 cells may possibly appear in a protective response to maintain epithelial homoeostasis (4). In fact, IL-17 production was induced by keratinocytes in an *in vitro* system (5). If the latter is the case, the more severe disruption in epithelial integrity in BP and PV, when compared with PF, may increase the number of regional Th17 cells.

The second intriguing issue is that the number of Foxp3+ cells in BP was significantly smaller than that in pemphigus groups. In fact, a decreased number or impaired functions of circulating Treg cells in several autoimmune diseases have been reported (6). Therefore, the decrease in Treg cells in BP is plausible. However, the different results between the BP and pemphigus groups cannot be explained. Treg cells were reported to be upregulated in pemphigus groups (2,7). One possibility is the difference in pathogenesis between pemphigus and BP; inflammation is more crucial for blister formation in BP than in pemphigus (8). Therefore, it is tempting to speculate that the involvement of an inflammatory milieu may decrease the number of Treg cells in BP skin lesions. In fact, it was reported that IL-6 secretion was upregulated, TGF-$\beta$ secretion was downregulated, and the number of circulating $\gamma\delta$ T cells was reduced in BP (9–11). The other possibility is repression of the migration of Treg cells into the inflammatory region in BP, but it may not be the case, because Treg chemoattractants were observed in both the affected skin and the bullae in BP (12,13). Previously, another group suggested that Treg cells accumulated in BP or MMP tissue (14,15). The discrepancy between our results and theirs is unexplained, but the genetic or racial differences maybe involved.

Our studies showed clear differences in the status of lesional effector/regulatory T-cell subsets between BP and pemphigus, although this study was not conducted in a perfectly blinded fashion. The results in the present study provide clues to elucidation of the pathogenesis of BP.

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## Conflict of interest

There is no conflict of interest.

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## Supporting Information

Additional Supporting Information may be found in the online version of this article:

**Figure S1.** Correlation of lesional lymphocytes or eosinophils with various parameters.

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**e-Supplement Figure 1.** Correlation of lesional lymphocytes or eosinophils with various parameters. (a) Number of IL17-producing cells (IL-17)/number of CD4-positive cells (CD4) vs CD4, (b) number of Foxp3-positive cells (Foxp3)/CD4 vs CD4, (c) IL-17/CD4 vs age at onset, (d) Foxp3/CD4 vs age at onset, (e) IL-17/CD4 vs maximal dose of prednisolone (PSLmax), (f) Foxp3/CD4 vs PSLmax, (g) IL-17/CD4 vs titer of BP180 ELISA indexes (BP180), (h) Foxp3/CD4 vs BP180, (i) IL-17/CD4 vs severity index, (j) Foxp3/CD4 vs severity index, (k) number of eosinophils (Eos) vs IL-17/CD4, (l) Eos vs Foxp3/CD4 and (m) Eos vs severity index.