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CUTTING EDGE

Cutting Edge: Human Th17 Cells Are Identified as Bearing CCR2$^+$CCR5$^-$ Phenotype[1]

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Recent reports have shown that IL-17-producing CD4$^+$ T cells (Th17 cells) belong to a distinct helper T cell lineage and are critically involved in the pathogenesis of autoimmune diseases and allergies. However, the chemokine receptor profile of Th17 cells remains to be clarified. In this study, we report that human Th17 cells are identified as CCR2$^+$CCR5$^-$ memory CD4$^+$ T cells. Analysis of PBMC from healthy donors showed that CCR2$^+$ cells produce much larger amounts of IL-17 than CCR2$^-$ cells, indicating the preferential expression of CCR2 on Th17 cells. Notably, CCR2$^+$CCR5$^-$ memory CD4$^+$ T cells produced a large amount of IL-17 and little IFN-γ, whereas CCR2$^+$CCR5$^+$ cells reciprocally produced an enormous amount of IFN-γ but little IL-17. Moreover, a higher expression of T-bet was seen in the CCR5$^+$ memory T cells. These results indicate that absence of CCR5 distinguishes human Th17 cells from Th1 cells. The Journal of Immunology,* 2007, 178: 7525–7529.

CD4$^+$ Th cells are essential regulators of adaptive immune responses. Th cells have been classified as either Th1 or Th2 according to the cytokine production profile and functional properties. However, recent studies have demonstrated that IL-17-producing T cells, rather than Th1 cells, play a pivotal role in the pathogenesis of autoimmune disease models, including experimental autoimmune encephalomyelitis (EAE)[3] (1–4). IL-17 is a cytokine mainly produced by activated memory T cells and could recruit and expand neutrophils through induction of various chemokines and GM-CSF (5–7).

Numerous studies have provided evidence that IL-17-producing T cells belong to a distinct lineage of Th cells whose development is severely hampered in IL-23 knockout but not in IL-12 knockout mice (8, 9). Although IL-23 was initially thought to induce differentiation of the IL-17-producing cells, it now seems that IL-23 is not involved in differentiation but propagation of Th17 cells (10). In fact, recent studies have shown that a combination of TGF-β1 plus IL-6 promotes the differentiation of Th17 cells in vitro (11–13). Differentiation of Th17 cells is prohibited by IFN-γ or IL-4 (11–13), further supporting the concept that Th17 cells comprise a distinct population cross-regulated by Th1 or Th2 cells. Notably, the independent nature of Th17 cells has been further highlighted by the recent discovery that the transcription factor RORγt is critically involved in the development of Th17 cells (14).

During the critical process whereby naive CD4$^+$ T cells differentiate, they acquire reciprocal sets of chemokine receptors (15), which would endow them a unique character of homing or migration to corresponding ligand chemokines. Namely, Th1 cells preferentially express CCR5 and CXCR3 and migrate to inflammatory milieu expressing the corresponding ligand chemokines, whereas Th2 cells express CCR4, CCR8, and CRTh2 indicative of a distinctive homing property (16–19). It is conceivable that Th17 cells may also possess unique chemotactic and migratory property. However, chemokine receptor expression by Th17 cells has not been characterized yet, at least to our knowledge.

In this study, we attempted to identify chemokine receptor expression by human Th17 cells by examining cytokine production profiles of T cell subpopulation-bearing chemokine receptor(s) of interest (16, 20). We started by comparing CCR2$^+$ and CCR2$^-$ memory CD4$^+$ T cells, because CCR2 and its ligand CCL2 were shown to be essential for development of EAE (21, 22). We found that only the CCR2$^+$ subpopulation would produce IL-17. Further analysis has demonstrated that CCR5$^-$ cells among the CCR2$^+$CD4$^+$ memory T cells produce IL-17, whereas a CCR5$^+$ subpopulation produces IFN-γ. Thus, human Th17 cells are identified as uniquely bearing the CCR2$^+$CCR5$^-$ phenotype.

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[3] Abbreviations used in this paper: EAE, experimental autoimmune encephalomyelitis; DN, double negative; DP, double positive; SP, single positive; MFI, mean fluorescence intensity.

Materials and Methods

Reagents

Anti-CCR2-biotin mAb, anti-CCR5-FITC mAb, and goat anti-IL-23R polyclonal Abs were purchased from R&D Systems. Streptavidin-PE, anti-CD4-PC5 mAb, and anti-CD45RA-energy-coupled dye (ECD) mAb were obtained from Beckman Coulter, anti-CCR5-allophycocyanin mAb from BD Pharmingen, and donkey anti-goat IgG-FITC from Jackson ImmunoResearch Laboratories. Anti-T-bet mAb and isotype control Ab (mouse IgG1a) purchased from Santa Cruz Biotechnology were used for intracellular staining. RPMI 1640 medium (Invitrogen Life Technologies) was supplemented with 0.05 mM 2-ME, 2 mM L-glutamine, 100 U/ml penicillin/streptomycin, and 10% FBS.

Cell preparation

Peripheral blood was obtained from healthy human volunteers (24–42 years of age) from whom informed consent was obtained. The Ethics Committee of the National Center of Neurology and Psychiatry approved the study. PBMC were freshly isolated by density gradient centrifugation using Ficoll-Paque Plus (Amersham Biosciences). To purify whole T cells or memory $CD4^+$ T cells from PBMC, we used a Pan T cell isolation kit II or Memory $CD4^+$ T cell isolation kit (Miltenyi Biotec), respectively. Briefly, PBMC were labeled with a mixture of biotin-conjugated mAbs directed against either non-T or non-memory $CD4^+$ T cells and then reacted with magnetic microbead-conjugated anti-biotin mAbs. The magnetically labeled non-T or non-memory $CD4^+$ T cells were depleted with autoMACS (Miltenyi Biotec), which yielded >95% purity of whole T cells or memory $CD4^+$ T cells as assessed by flow cytometry for the proportion of $CD3^+$ cells or $CD4^+CD45RA^-$ cells.

To further separate the purified cells according to CCR2 or CCR5 expression, they were labeled with anti-CCR2-biotin, streptavidin-PE, and anti-PE microbeads (Miltenyi Biotec) or anti-CCR5-FITC and anti-FITC microbeads (Miltenyi Biotec). The magnetically labeled cells were separated into positive ($CCR2^+$ and $CCR5^+$) and negative ($CCR2^-$ and $CCR5^-$) fractions with autoMACS (>99% purity of $CCR2^-$ or $CCR5^-$ cells and >90% purity of $CCR2^+$ or $CCR5^+$ cells). To obtain $CCR2^+CCR5^-$ and $CCR2^+CCR5^+$ memory $CD4^+$ T cells, $CCR2^+$ memory $CD4^+$ T cells were labeled with anti-CCR5-allophycocyanin and separated into $CCR2^+CCR5^-$ (>80% purity) and $CCR2^+CCR5^+$ cells (>95% purity) by flow cytometric cell sorter Epics Altra (Beckman Coulter).

Cell culture and cytokine measurement by ELISA

Purified T cell populations were resuspended at 5×10^5/ml and stimulated with PMA (50 ng/ml) and ionomycin (1 μg/ml) in 96-well U-bottom plates for 24 h. The concentrations of IFN-γ and IL-17 in the supernatants were measured by using a Human IFN-γ ELISA Set (BD Pharmingen) and a Human IL-17 DuoSet (R&D Systems).

Flow cytometric analysis of chemokine receptors

To evaluate the expression of chemokine receptors, purified memory $CD4^+$ T cells were stained with anti-CD4-PC5, anti-CD45RA-ECD, anti-CCR5-FITC and PE-conjugated mAbs against anti-CCR2-biotin were analyzed with Epics flow cytometry (Beckman Coulter). To examine the expression of IL-23R, memory $CD4^+$ T cells were stained with goat anti-IL-23R and anti-goat-IgG-FITC and were analyzed with a FACSCalibur (BD Pharmingen).

Intracellular staining of T-bet

Purified memory $CD4^+$ T cells were first stained with biotin-conjugated anti-CCR2, streptavidin-PE, and allophycocyanin-CCR5, then fixed in PBS containing 2% paraformaldehyde and permeabilized with 0.1% saponin solution. Subsequently, the cells were stained with FITC-anti-T-bet. Mouse IgG1a was used as an isotype control.

Statistics

An unpaired Student's *t* test or one-way ANOVA was used for statistical analysis. We considered $p < 0.01$ as significant.

Results and Discussion

Both Th17 cells and Th1 cells are enriched in $CCR2^+$ $CD4^+$ memory T cells

Previous reports on the CCR2 requirement for development of EAE (21, 22) prompted us to compare the cytokine-producing ability of $CCR2^+$ and $CCR2^-$ cells isolated from whole T cells. The results showed that $CCR2^+$ cells produced a larger amount of IFN-γ and IL-17 as compared with $CCR2^-$ cells, whereas unseparated whole T cells showed intermediate values (Fig. 1*A*, *upper panels*). This indicates that $CCR2^+$ cells contain the vast majority of Th1 and Th17 cells. We next separated the whole T cells into $CCR5^+$ and $CCR5^-$ populations to compare the cytokine profile. Although $CCR5^+$ cells produced a larger amount of IFN-γ as compared with $CCR5^-$ or the whole T

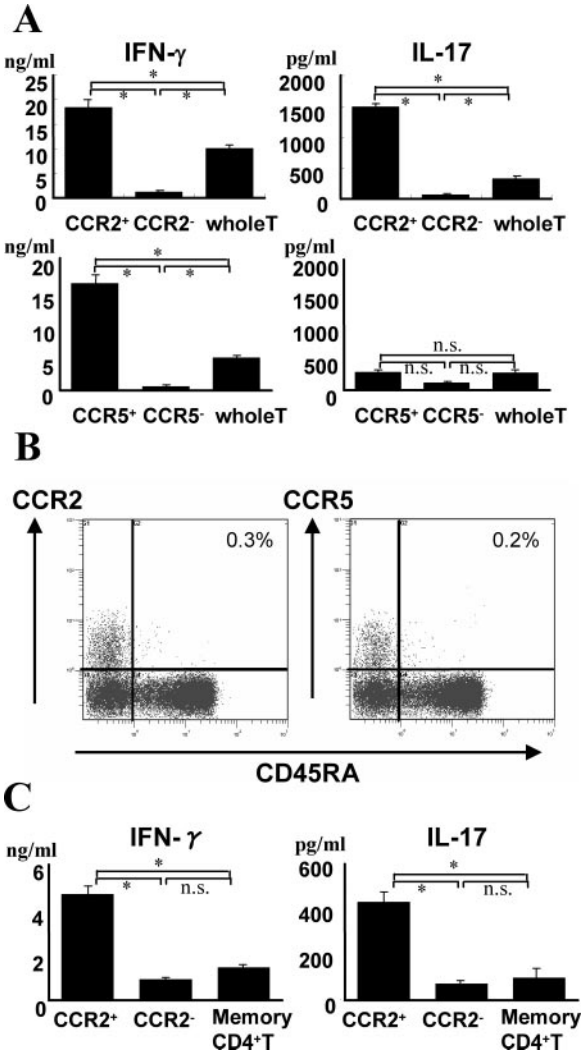


FIGURE 1. Th17 cells are enriched in $CCR2^+CD4^+CD45RA^-$ cells. *A*, IL-17-producing cells are selectively enriched in the $CCR2^+$, but not in a $CCR5^+$ population. From PBMC of healthy donors, whole T cells were purified magnetically by negative selection using a Pan T cell isolation kit II with autoMACS. The purity of the cells was generally >98%, as determined by FACS analysis. Purified $CCR2^+$ and $CCR2^-$ T cells (or $CCR5^+$ and $CCR5^-$ T cells) were stimulated with PMA and ionomycin for 24 h before the supernatants were collected. The IFN-γ and IL-17 protein in each supernatant was measured using ELISA. Results are expressed as mean ± SD of a representative of five independent experiments. *B*, Chemokine receptor (CCR2 or CCR5) expressing T cells are largely confined to $CD45RA^-$ memory T cells. PBMC from healthy subjects were stained with anti-CCR2 (PE), anti-CCR5 (FITC), anti-CD4 (PC5), and anti-CD45RA (ECD) and analyzed after being gated for CD4. Shown is a representative of five individual data sets. *C*, Th17 cells are enriched in $CCR2^+CD4^+CD45RA^-$ cells. Memory $CD4^+$ T cells were purified by a memory $CD4^+$ T cell isolation kit with autoMACS. $CCR2^+$ and $CCR2^-$ T cells were further isolated by anti-CCR2-biotin, streptavidin-PE, anti-PE microbeads, and autoMACS. Purified cells were stimulated with PMA and ionomycin for 24 h before collecting supernatants. Results are expressed as mean ± SD of a representative of five independent experiments. *, $p < 0.01$.

cells (Fig. 1*A, lower panels*), production of IL-17 did not increase after enrichment for $CCR5^+$ cells. These results suggest that Th17 cells may be selectively enriched in $CCR2^+$, but not in $CCR5^+$ populations. However, because the $CCR2^+$ T cell preparation also contains $CD8^+$ T cells and γδT cells, capable of producing IL-17 (23–25), it remained possible that the major source of IL-17 could be $CD8^+$ T or γδT cells. Therefore, we next needed to assess the production of IFN-γ and IL-17 from purified $CD4^+$ T cells. Preparatory experiments showed that $CCR2^+$ or $CCR5^+CD4^+$ populations are mainly confined to the $CD45RA^-$ memory T cell population (Fig. 1*B*). Consequently, we decided to use memory $CD4^+$ T cells that could be obtained after deleting $CD8^+$, γδ, and naive $CD4^+$ T cells for further analysis. Analysis of the purified memory $CD4^+$ T cells has also demonstrated that the $CCR2^+$ population produced a significantly larger amount of both IFN-γ and IL-17 compared to the $CCR2^-$ population, with the values of unseparated cells being intermediate (Fig. 1*C*). These results strongly indicate that Th17 cells as well as Th1 cells are enriched in $CCR2^+CD4^+$ memory T cells. However, since Th1 and Th17 cells are thought to belong to distinct T cell lineages, we speculated that they might be further divided into two subpopulations based on expression of chemokine receptors.

$CCR2^+CCR5^-$ memory $CD4^+$ T cells predominantly produce IL-17 but not IFN-γ

Simultaneous staining of CCR2 and CCR5 showed that the $CCR2^+$ memory T cell population could be divided into $CCR5^-$ (CCR2 single positive (SP)) and $CCR5^+$ (CCR2 and CCR5 double positive (DP)) subpopulations (Fig. 2). Since CCR5 is reported to be expressed predominantly on Th1 cells (16–18), we hypothesized that SP and DP cells might correspond to Th17 and Th1 cells, respectively. To correlate cytokine production profile and chemokine receptor expression in T cell populations, we first thought of staining total unseparated T cells to detect intracellular cytokines as well as surface CCR; however, the cell activation process required for intracellular cytokine staining was found to down-regulate CCR2 and CCR5 significantly (data not shown), as reported previously (26). To accurately correlate the expression of CCR2 or CCR5 with the cytokine profile ex vivo, we decided to first isolate SP and DP cells from memory $CCR2^+$ T cells by using a flow cytometric cell sorter and stimulate them with PMA and ionomycin. We then measured IFN-γ and IL-17 in the supernatant (Fig. 3*A*). Remarkably, the sorted T cell subpopulations exhibited different cytokine production patterns: SP cells produced a large amount of IL-17 and a small amount of IFN-γ, whereas DP cells produced a small amount of IL-17 and a large amount of IFN-γ (Fig. 3*B*). These results suggest that Th17 cells are largely confined to SP cells, whereas DP cells contain a majority of Th1 cells.

T-bet and IL-23R expression in memory $CD4^+$ T cells

Finally, we assessed whether SP and DP cells are distinctive in expression of transcription factor T-bet and IL-23R. T-bet is an essential transcription factor for Th1 differentiation (27), whereas it was reported to be redundant for Th17 cells (3, 8, 9, 11, 14). IL-23 has been shown to play a pivotal role in the survival and expansion of Th17 cells (2, 10). Magnetically purified memory $CD4^+$ T cells were first stained with biotin-conjugated CCR2, streptavidin-PE, and allophycocyanin-CCR5, and then were intracellularly stained with FITC-anti-T-bet or were stained with goat anti-IL-23R Ab and anti-goat IgG-FITC. We compared T-bet expression in SP vs DP cells by evaluating the mean fluorescence intensity (MFI) (Fig. 4, *A* and *B*). T-bet was significantly expressed by SP as well as $CCR2^-CCR5^-$ double-negative (DN) cells, but its expression was much higher in DP cells and $CCR2^-CCR5^+$ cells, suggesting that Th1 cells may be confined to $CCR5^+$ populations. On the other hand, the frequency of $IL\text{-}23R^+$ cells was highest in the SP fraction, compared with the others (Fig. 4, *C* and *D*).

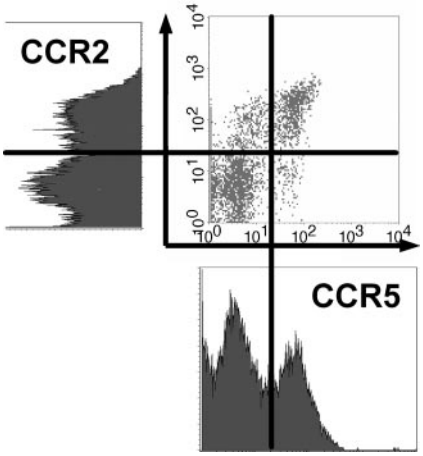


FIGURE 2. $CCR2^+$ memory $CD4^+$ T cells consist of $CCR5^+$ and $CCR5^-$ subsets. Purified memory $CD4^+$ T cells were stained with anti-CCR2-biotin and streptavidin-PE as well as anti-CCR5-FITC. The separation of positive and negative populations was determined by histogram plots.

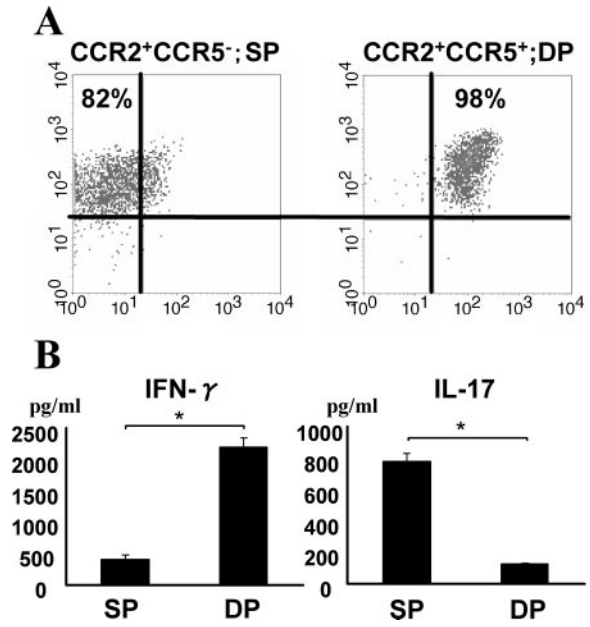


FIGURE 3. $CCR2^+CCR5^-$ (SP) and $CCR2^+CCR5^+$ (DP) cells correspond to Th17 and Th1 cells, respectively. SP and DP subsets were sorted from memory $CD4^+$ T cells by flow cytometry and stimulated with PMA/ionomycin. *A*, A representative of five individual data sets showing the purity of the sorted cells. *B*, SP and DP cells were stimulated with PMA/ionomycin for 24 h. Then the amounts of IFN-γ and IL-17 in the supernatant were measured using ELISA. Results are expressed as mean ± SD of a representative of five independent experiments. *, $p < 0.01$

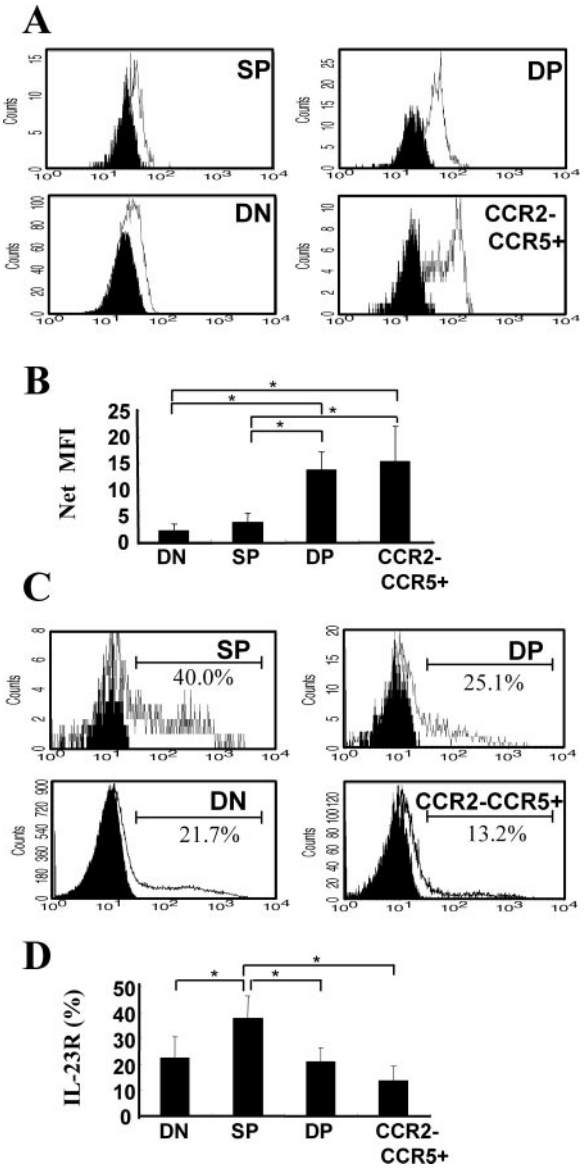


FIGURE 4. T-bet expression of memory $CD4^+$ T cells. *A*, Histogram plots of T-bet expression within memory $CD4^+$ T cells. SP, DP, DN ($CCR2^-CCR5^-$), and $CCR2^-CCR5^+$ T cells were stained with anti-T-bet or isotype control Ab. Shown is a representative of five individual data sets. *B*, The MFI of each histogram plot. Data for the MFI of T-bet expression subtracted by that of control Ab are calculated and shown as bar graphs with error bars showing the SD of four individual data sets. *C*, Histogram plots of IL-23R expression within memory $CD4^+$ T cells. The cells were stained with goat anti-IL-23R polyclonal Ab and anti-goat-FITC Ab. A representative of seven individual data sets is shown. *, $p < 0.01$. *D*, The frequency of IL-23R-positive cells of each histogram plot. Data are shown as bar graphs with error bars showing the SD of seven individual data sets. *, $p < 0.01$.

Given the distinguished ability to produce IL-17 as well as higher IL-23R and lower T-bet expression, we propose that Th17 cells are confined to SP cells, whereas Th1 cells are either DP or $CCR2^-CCR5^+$. It has recently been reported that T-bet directly regulates the transcription of IL-23R in mice (28). It is possible that weak expression of IL-23R by non-Th17 cells (DP, DN, and $CCR2^-CCR5^+$) may result from baseline activation of T-bet.

Additional remarks

Using freshly isolated healthy human lymphocytes, we showed here that $CCR2^+CCR5^-$ memory T cells would produce a large amount of IL-17 but not IFN-γ, whereas $CCR2^-$ memory T cells produced IFN-γ, but not IL-17. Although we presented the data obtained after stimulation with PMA/ionomycin, polyclonal stimulation by anti-CD3/CD28 also gave similar results (data not shown). Moreover, when we stimulated $CCR2^+CD4^+$ memory T cells by IFN-γ, IL-4, IL-2, or IL-23 in addition to PMA/ionomycin, IL-17 production was not changed (data not shown).

The frequency of Th17 cells among this subset is an important issue to be investigated. By using the ELISPOT assay, we found that ~200 spots of IL-17-producing cells could be detected among 1×10^5 memory $CCR2^+CD4^+$ T cells (~0.2%), whereas the numbers of IFN-γ-producing cells were about 5-fold higher (~1.0%). Although this needs to be systematically verified, the lower frequency of IL-17-producing cells is consistent with the lower value of IL-17 than IFN-γ in supernatants detected by ELISA.

The unique chemokine receptor expression pattern of Th17 cells provides a basis for their recruitment to specialized inflammatory conditions in vivo, which should be relevant for understanding the pathogenesis of autoimmune diseases.

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Disclosures

The authors have no financial conflict of interest.

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