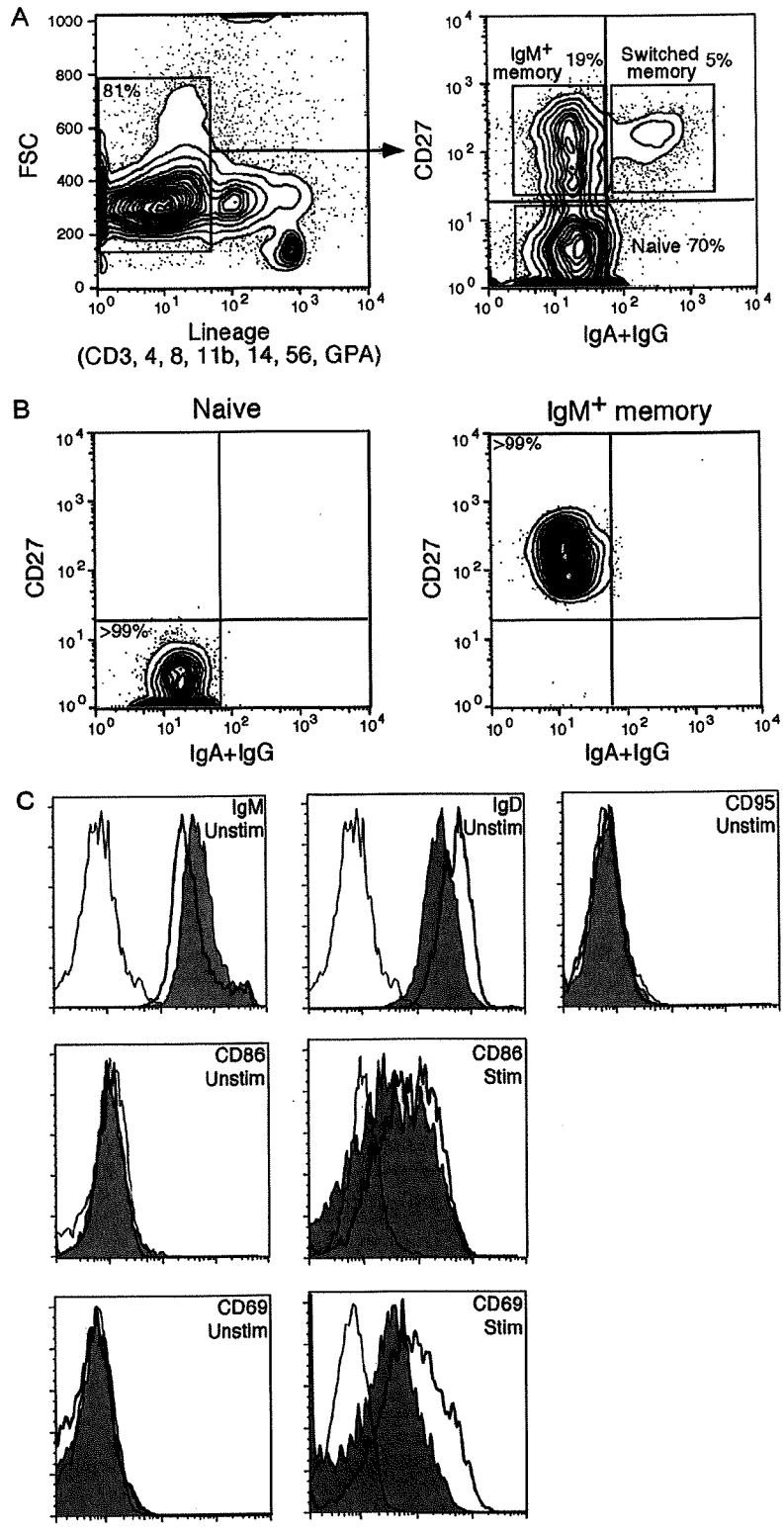

FIGURE 1. Isolation of purified human peripheral B cell subsets. *A*, Phenotypic analysis of B cell subsets in human peripheral blood. Donor B cells were purified by staining with Abs to CD3, CD4, CD8, CD11b, CD14, CD56, and glycophorin A (GPA) and were then evaluated by flow cytometry. B cell subsets were identified according to surface IgG/IgA and CD27 expression: $IgG^-A^-CD27^-$ B cells (naive), $IgG^-A^-CD27^+$ B cells (IgM^+ memory), and $IgG^+A^+CD27^+$ B cells (switched memory). Data are presented as density plots. *B*, Highly purified B cell subsets were separated after cell sorting. *C*, Surface marker expression in human B cell subsets. Purified B cell subsets before (Unstim) and after stimulation (Stim) with 20 μg/ml $F(ab')_2$ goat anti-hIgM (36 h) were analyzed separately for IgM, IgD, CD95, CD86, and CD69 surface expression. Bold line, Naive B cells; gray area, IgM^+ memory B cells; thin line, isotype control line. These results are representative of peripheral blood samples from more than 10 different donors.

QuantiTect reverse transcription kit (Qiagen). Quantitative real-time PCR was conducted in the ABI Prism 7700 Sequence Detector (Applied Biosystems). Reactions were performed in triplicate wells in 96-well plates. TaqMan target mixes for Bim, Bcl-x_L, Mcl-1, Tcl1, and Gal-1 were purchased from Applied Biosystems. 18S rRNA was separately amplified in the same plate to be used as an internal control for variances in the amount of cDNA in PCR. Collected data were analyzed with Sequence Detector software (Applied Biosystems). Data were expressed as a fold change in gene expression relative to those from unstimulated naive B cells.

Intracellular flow cytometry

After two washings with PBS containing 1% FCS, 5×10^5 cells were placed in a 96-well microtiter plate. Cells were resuspended with 50 μl of medium plus 50 μl of fixation buffer (BD Biosciences) and incubated for 10 min at 37°C. After washing again with PBS containing 1% FCS, cells were suspended with 50 μl of saponin permeabilization buffer (BD Biosciences) and spun down. The cell pellet was incubated with primary Abs (anti-human Mcl-1 or Bim) in saponin buffer at room temperature for

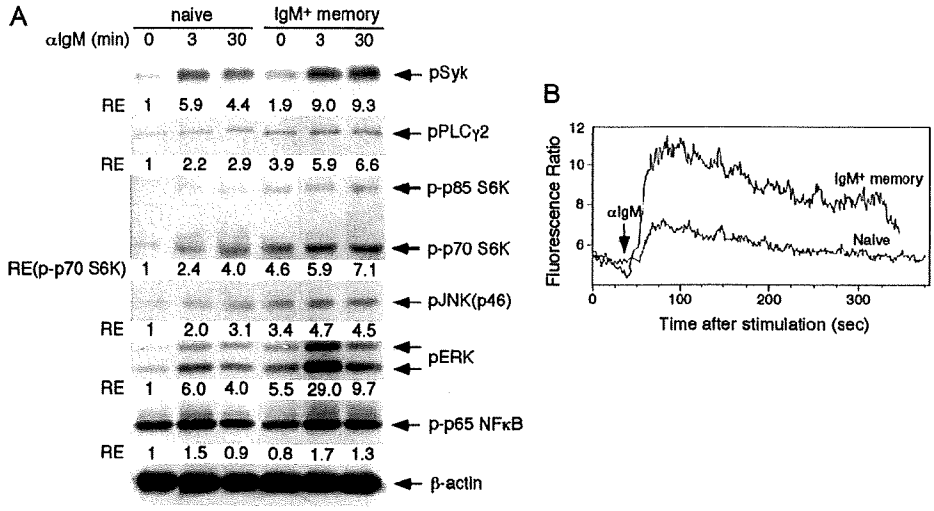

FIGURE 2. BCR signaling profiles of naive and IgM$^+$ memory B cells at early time points. *A*, B cell subsets were stimulated with 20 μg/ml F(ab')$_2$ goat anti-hIgM for the indicated time intervals. Cell lysates were subsequently separated on an 8% or 10% SDS-PAGE gel, and analyzed by Western blotting with anti-phospho-Syk, -PLCγ2, -JNK, -ERK sera, and anti-phospho-p70/85 S6K, -p65 NF-κB mAb and anti-β-actin mAb. Results are representative of three independent experiments. RE, Relative expression. *B*, Ca^{2+} mobilization in naive and IgM$^+$ memory B cells. Intracellular free calcium levels in fluo-4-acetoxymethyl ester-loaded cells were monitored using flow cytometry, after cells were stimulated with 20 μg/ml F(ab')$_2$ goat anti-hIgM. Results are representative of five independent experiments.

30–45 min. After a washing with PBS containing 1% FCS, cell were incubated with PE-conjugated donkey anti-rabbit IgG (Jackson Immuno-Research Laboratories) at room temperature for 30–45 min. Cells were washed one more time with PBS containing 1% FCS and analyzed at low flow rate on a FACSCalibur. B cell population was identified on its forward and side scatter distribution, and 15,000 cell events were analyzed for mean fluorescence using FlowJo software.

Results

Early BCR signaling is exaggerated in IgM$^+$ memory but not naive B cells

BCR signaling is critical for B cell fate decisions such as B cell survival, growth, and differentiation (14). We first tested whether the profile of early BCR signaling is different between naive and IgM$^+$ memory B cells. Phosphorylation of Syk, one of the earliest events in BCR signaling, was more pronounced in IgM$^+$ memory B cells (Fig. 2*A*). Two enzymes, PI3K and PLCγ2, function as critical mediators downstream of Syk activation in B cells (15). Phosphorylation of p85/p70 S6K, a downstream molecule of PI3K, and PLCγ2, was more pronounced in IgM$^+$ memory B cells (Fig. 2*A*). Activated PLCγ2 converts phosphatidylinositol 4,5-bisphosphate into IP3 and diacyl glycerol, the former of which is critical for calcium flux in B cells (14). Consistent with PLCγ2 phosphorylation, BCR-induced calcium flux was higher in IgM$^+$ memory B cells (Fig. 2*B*). Calcium flux and diacyl glycerol led to activation of NF-κB and MAPKs such as JNK and ERK. Phosphorylation of JNK and ERK was more pronounced in IgM$^+$ memory B cells, whereas p65 NF-κB phosphorylation was comparable in both subsets (Fig. 2*A*). Taken together, during the early phase of BCR activation, downstream signaling is pronounced especially in IgM$^+$ memory B cells as compared with naive B cells.

BCR stimulation rescues naive but not IgM$^+$ memory B cells from apoptosis

Following anti-IgM stimulation alone, naive and IgM$^+$ memory B cells did not either divide or release Igs in the culture (data not shown), suggesting that BCR signaling alone is not sufficient to induce the growth and differentiation of human B cell subsets. We then tested whether the BCR signaling affects the survival and death of naive and IgM$^+$ memory B cells. In the absence of stimuli, a considerable fraction of purified naive and IgM$^+$ memory B cells underwent apoptotic cell death within 2 days in vitro (Fig. 3*A*). Spontaneous cell death was more pronounced in naive B cells than in IgM$^+$ memory B cells. BCR stimulation, however, significantly rescued naive B cells from apoptosis, whereas IgM$^+$ memory B cells were not rescued (Fig. 3, *A* and *B*). Thus, BCR signaling can protect naive, but not IgM$^+$ memory B cells from apoptotic cell death.

Mitochondrial perturbations including cytochrome *c* release and inner membrane depolarization correlate with BCR-induced apoptosis (16). We thus tested whether BCR-induced depolarization of the mitochondrial inner membrane could be altered in naive and IgM$^+$ memory B cells. High levels of mitochondrial membrane potential were observed in both subsets immediately after sorting, indicating their highly viable state (Fig. 3*C*, *a* and *d*). A 2-day culture of these subsets without stimuli caused a remarkable decrease in mitochondrial membrane potential (Fig. 3*C*, *b* and *e*). BCR stimulation for 2 days, however, partially abrogated the loss of mitochondrial membrane potential in naive, but not IgM$^+$ memory B cells (Fig. 3*C*, *c* and *f*). Thus, BCR signaling rescues the B cell apoptosis pathway upstream of mitochondrial damage in naive, but not IgM$^+$ memory B cells.

BCR stimulation induces anti-apoptotic Mcl-1 in naive B cells, whereas it induces proapoptotic Bim in IgM$^+$ memory B cells at the protein level

Bcl-2 family proteins are the primary regulators of mitochondrial membrane integrity and play a vital role in the control of apoptosis (9). We tested whether BCR signaling affects gene expression of Bim, Bcl-x_L, and Mcl-1 in naive and IgM$^+$ memory B cells (Fig. 4*A*). Bcl-x_L mRNA expression was induced after BCR stimulation in both subsets, and such induction was more pronounced in naive B cells. In contrast, the expression level of Bim mRNA was slightly higher in IgM$^+$ memory B cells irrespective of BCR stimulation. Mcl-1 mRNA expression was not significantly changed in both subsets. We next tested whether BCR signaling affects protein

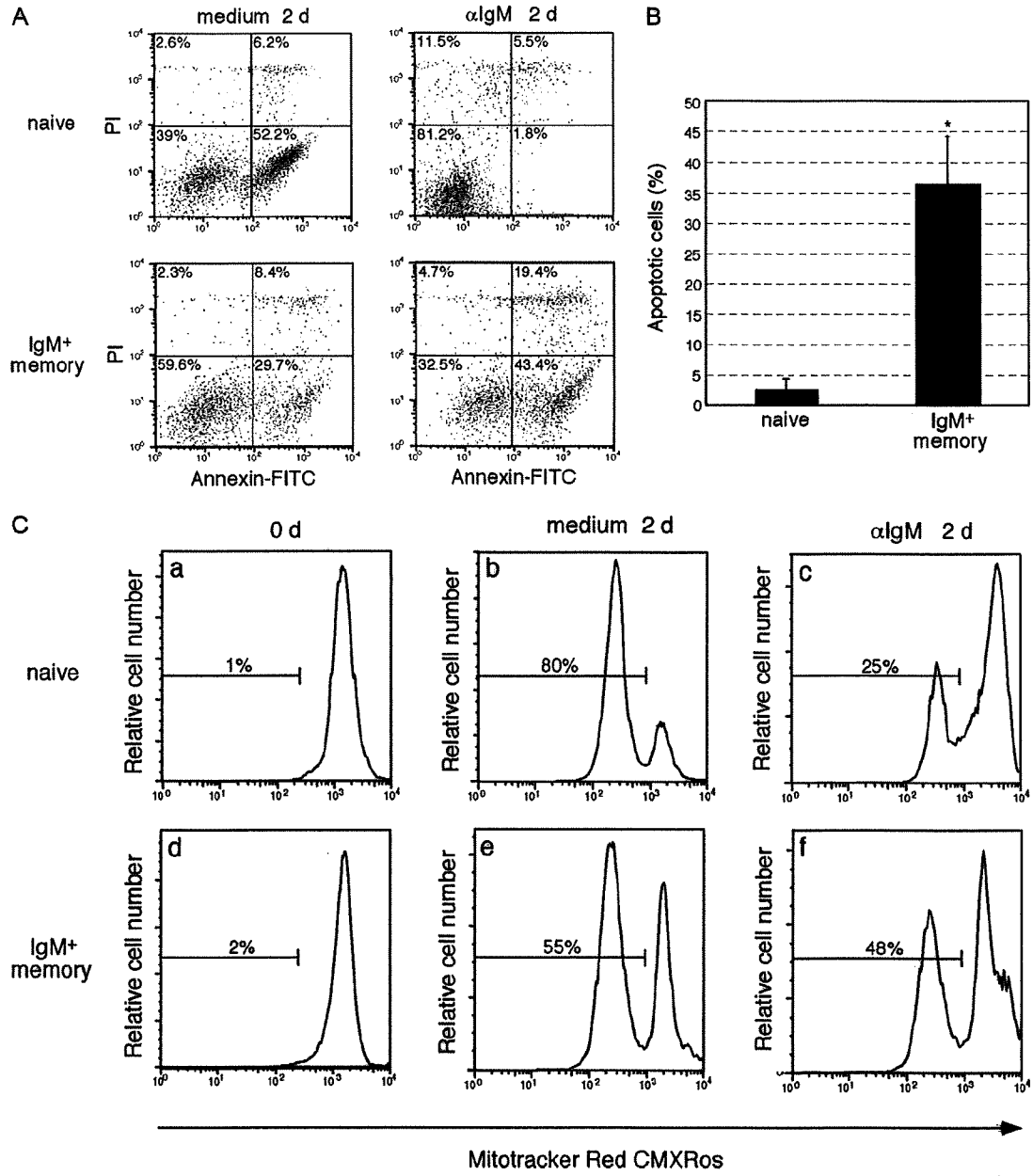

FIGURE 3. BCR-induced apoptosis in B cell subsets. *A*, Naive and IgM$^+$ memory B cells were incubated in the absence or presence of 20 μg/ml F(ab')$_2$ goat anti (α)-hIgM. After 2 days (2 d) of culture, cells were double-stained with FITC-labeled annexin V and propidium iodide (PI) and analyzed using flow cytometry. Apoptotic cells are visible as annexin V-positive population, both propidium iodide negative (early apoptosis) and propidium iodide positive (late apoptosis). The results shown are representative of three independent experiments. *B*, Percentage apoptosis of B cell subsets after BCR stimulation. The data are shown as the mean ± SD of six independent experiments. *, $p < 0.01$. *C*, BCR-induced mitochondrial inner membrane depolarization in B cell subsets. Naive and IgM$^+$ memory B cells were treated for 2 days with either medium or 20 μg/ml F(ab')$_2$ goat anti-human IgM. Percentages of Mitotracker Red CMXRos^{low} cells are shown. Data obtained from cells immediately after isolation are also shown. Results are representative of three independent experiments.

expression of Bim, Bcl-x_L, and Mcl-1 in naive and IgM$^+$ memory B cells (Fig. 4*B*). Three isoforms (Bim-EL, Bim-L, and Bim-S) are expressed in various cell types, including lymphocytes (17). In the absence of stimuli, Bim-EL was weakly expressed in both subsets, but in IgM$^+$ memory B cells BCR stimulation induced all of three Bim isoforms at the level higher than those in naive B cells. On the other hand, the expression level of anti-apoptotic proteins Bcl-x_L and Mcl-1 was higher in naive B cells after BCR stimulation. Given that the difference in expression levels of surface IgM between two subsets (Fig. 1*C*) might cause these phenomena, we tested Mcl-1 expression in naive and IgM$^+$ memory B cells using titrated doses of anti-IgM Ab. Higher levels of Mcl-1 in naive B cells were observed at all doses of anti-IgM tested (Fig. 4*C*). Collectively, these results suggest that after BCR stimulation, anti-apoptotic Bcl-x_L and Mcl-1 are predominantly expressed in naive B cells, whereas the proapoptotic protein Bim was more abundantly expressed in IgM$^+$ memory B cells. The discrepancy of mRNA and protein levels strongly suggests the existence of post-transcriptional regulation of Bim and Mcl-1 expression in both subsets.

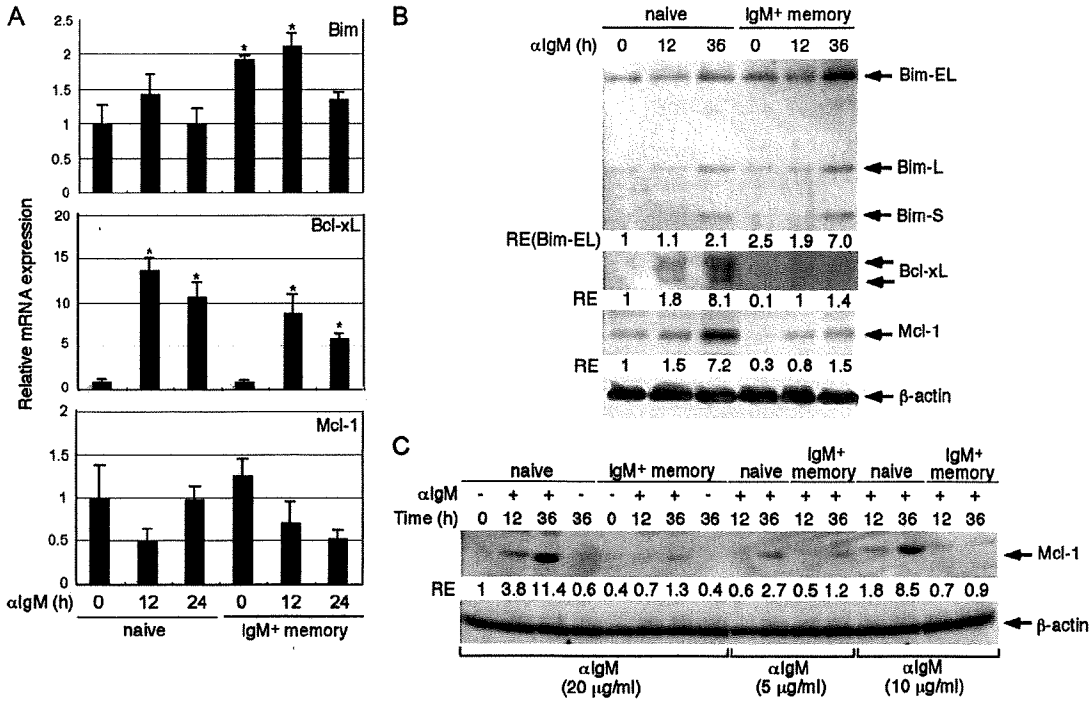

FIGURE 4. BCR-induced expression of Bcl-2 family proteins in B cell subsets. Naive and IgM^+ memory B cells were incubated for the indicated time periods in the absence or presence of 20 mg/ml $F(ab')_2$ goat anti (α)-hIgM. *A*, Quantitation of Bim, Bcl-x_L, and Mcl-1 mRNA by real-time PCR in B cell subsets. Data are normalized to the expression of 18S rRNA. Results are representative of three independent experiments. *, $p < 0.01$. *B*, Cell lysates were separated on a 12.5% SDS-PAGE gel, and analyzed by Western blotting with anti-Bim and -Mcl-1 sera and anti-Bcl-x_L and -β-actin mAb. The results shown are representative of five independent experiments. RE, Relative expression. *C*, Effect of a titrated dose of anti-IgM on Mcl-1 induction in B cell subsets. Results are representative of three independent experiments. RE, relative expression.

PI3K activation plays a role in reciprocal regulation of Mcl-1 and Bim protein in B cell subsets

A previous report suggests a critical role of the PI3K pathway in Mcl-1 expression (18). We tested an effect of a selective PI3K inhibitor Ly294002 on BCR-induced Mcl-1 expression in naive B cells (Fig. 5*A*). Treatment of cells with Ly294002 strongly inhibited Mcl-1 induction in naive B cells, suggesting a critical role of PI3K activity in Mcl-1 expression in naive B cells. In contrast, Ly294002 treatment induced the expression of Bim-EL protein after BCR stimulation (Fig. 5*A*). These results suggest reciprocal Mcl-1 and Bim expression is dictated by the PI3K pathway. We thus monitored the phosphorylation of Akt, a downstream molecule of PI3K, during BCR stimulation. Akt phosphorylation was sustained for longer periods in naive B cells than IgM^+ memory B cells (Fig. 5, *B* and *C*). A previous study showed that PI3K activation is critical for BCR-mediated induction of CD86 and CD69 surface expression in murine B cells (19). As shown in Fig. 1*C*, expression levels of CD86 and CD69 are higher in naive than IgM^+ memory B cells at a late time point. Collectively, these results suggest that reciprocal expression of Mcl-1 and Bim protein in both subsets could be explained by their distinct regulation of the PI3K pathway.

Tcl1 and Gal-1 are the critical mediators for B cell to express Mcl-1 and Bim proteins, respectively

To identify the molecule responsible for sustained activation of the PI3K pathway, we conducted gene expression profiling of B cell subsets before and after BCR stimulation. A subset of genes displayed >2-fold differences between naive and IgM^+ memory B cells (data not shown). Among these genes, we focused on Tcl1, a potent Akt kinase coactivator (20, 21). We tested Tcl1 mRNA expression in both subsets (Fig. 6*A*). In the absence of stimuli, a higher level of Tcl1 mRNA was observed in naive B cells than in IgM^+ memory B cells. BCR stimulation resulted in more than 10-fold mRNA induction of Tcl1 in naive B cells. We next evaluated the level of Tcl1 protein in both subsets (Fig. 6*B*). Tcl1 protein was detected only in naive B cells irrespective of stimulation. Reduction in the expression of Tcl1 protein implies the existence of a posttranscriptional inhibitory mechanism of Tcl1 expression. To determine whether Tcl1 expression can induce Mcl-1 expression to promote B cell survival, Tcl1 transgene was overexpressed in IgM^+ memory B cells. Enforced Tcl1 expression induced a high level of Mcl-1 expression in IgM^+ memory B cells and protected their apoptotic cell death (Fig. 6, *C* and *D*). Thus, Tcl1 expression in naive, but not IgM^+ memory B cells plays a critical role in Mcl-1 expression that in turn promotes their survival.

We also sought to identify a molecule involved in Bim expression and apoptosis in IgM^+ memory B cells. In a list of genes identified in microarray analysis, higher levels of a glycoprotein Gal-1 in IgM^+ memory B cells were noted (data not shown). In the absence of stimuli, Gal-1 mRNA was more expressed in IgM^+ memory B cells and BCR stimulation of this subset caused drastic mRNA induction of this gene (Fig. 7*A*). Consistent with its mRNA expression, Gal-1 protein was abundantly expressed in IgM^+ memory B cells (Fig. 7*B*). To test whether Gal-1 expression can induce Bim expression in B cells and enhance their apoptosis, *Gal-1* transgene was overexpressed in naive B cells. Enforced *Gal-1* expression resulted in higher levels of Bim expression in naive B cells, which is associated with the increment of apoptotic cells by >2-fold (Fig. 7, *C* and *D*). These results suggest that Gal-1 plays a vital role in promoting Bim expression and inducing apoptosis in IgM^+ memory B cells. Interestingly, Gal-1 also functions

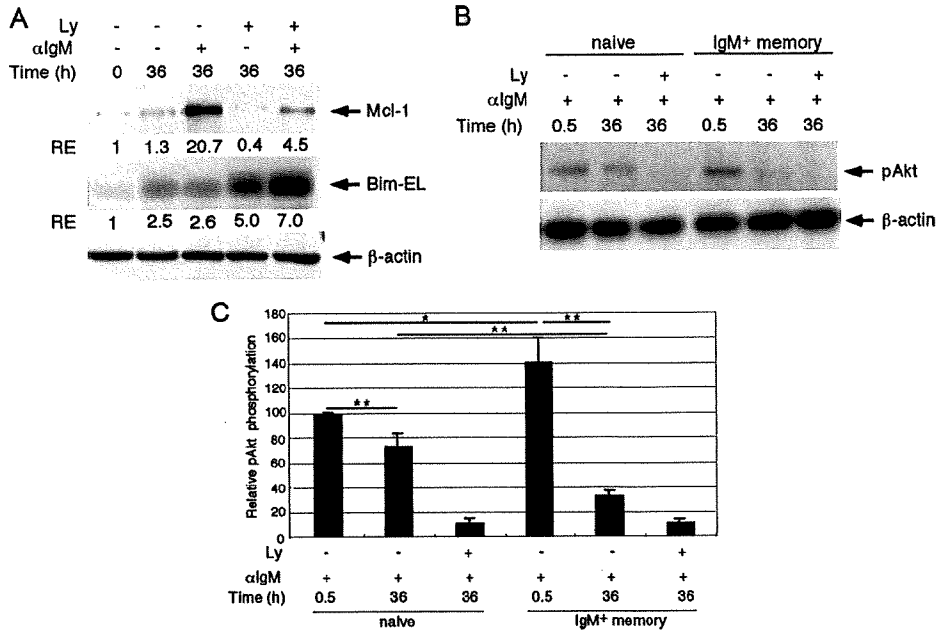

FIGURE 5. Regulation of Mcl-1 and Bim protein expression by the PI3K pathway. *A*, Naive B cells were pretreated with or without Ly294002 (Ly; 10 μM) for 30 min and stimulated for 36 h in the absence or presence of 20 μg/ml F(ab')$_2$ goat anti (α)-hIgM. Cell lysates were separated on a 12.5% SDS-PAGE gel, and analyzed by western blotting with anti-Bim, -Mcl-1 sera, and anti-β-actin mAb. The results shown are representative of three independent experiments. *B*, Naive and IgM$^+$ memory B cells were pretreated with or without Ly294002 (10 μM) for 30 min and stimulated for the indicated time periods in the absence or presence of 20 μg/ml F(ab')$_2$ goat anti-human IgM. Cell lysates were separated on a 10% SDS-PAGE gel and analyzed by Western blotting with anti-phospho-Akt sera and anti-β-actin mAb. Results are representative of three independent experiments. *C*, Densitometric analyses of Akt phosphorylation in B cell subsets. The resulting values were expressed as the percentage in reference to that of BCR-stimulated naive B cells at 0.5 h. Values are the mean ± SD of three independent experiments. *, $p < 0.05$; **, $p < 0.01$.

as a soluble cytokine (22). Because activation of Akt and JNK is critical for regulating Bim expression (23, 24), we tested the effect of recombinant Gal-1 on BCR-induced phosphorylation of Akt and JNK in B cells. As shown in Fig. 7*E*, Gal-1 remarkably inhibited Akt phosphorylation, whereas it slightly enhanced JNK phosphorylation in B cells upon BCR stimulation. Taken together, Gal-1 regulates Bim expression through its effects on activation of Akt and JNK in B cells.

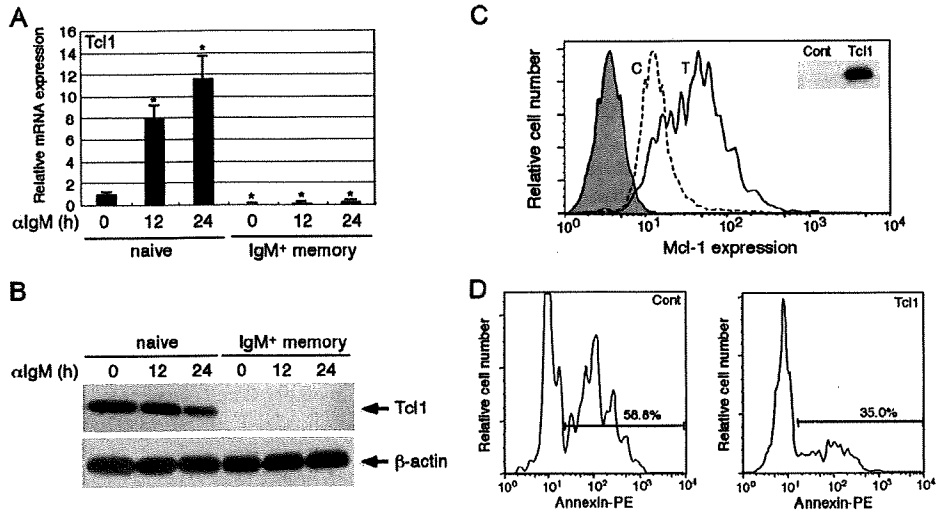

FIGURE 6. Tcl1 is critical for Mcl-1 expression and survival of B cell subsets. Naive and IgM$^+$ memory B cells were incubated for the indicated time intervals in the absence or presence of 20 μg/ml F(ab')$_2$ goat anti (α)-hIgM. *A*, Quantitation of Tcl1 mRNA by real-time PCR in naive and IgM$^+$ memory B cells. Data are normalized to the expression of 18S rRNA. Results are representative of five independent experiments. *, $p < 0.01$ (with reference to unstimulated naive B cells). *B*, Cell lysates were separated on a 12.5% SDS-PAGE gel, and analyzed by Western blotting with anti-Tcl1 sera, and anti-β-actin mAb. Results are representative of four independent experiments. IgM$^+$ memory B cells were transfected with either pEGFP-empty or -Tcl1 for 18 h and then stimulated with 20 μg/ml F(ab')$_2$ goat anti-hIgM for 24 h. *C*, After culture, intracellular Mcl-1 expression of GFP-positive cells was analyzed by flow cytometry. *Insets*, Expression of Tcl1 transgene. Results are representative of three independent experiments. *D*, After culture, GFP-positive cells were stained with PE-labeled annexin V and analyzed using flow cytometry. Percentages of annexin-positive cells are shown. Results are representative histogram of three independent experiments. Cont, Control.

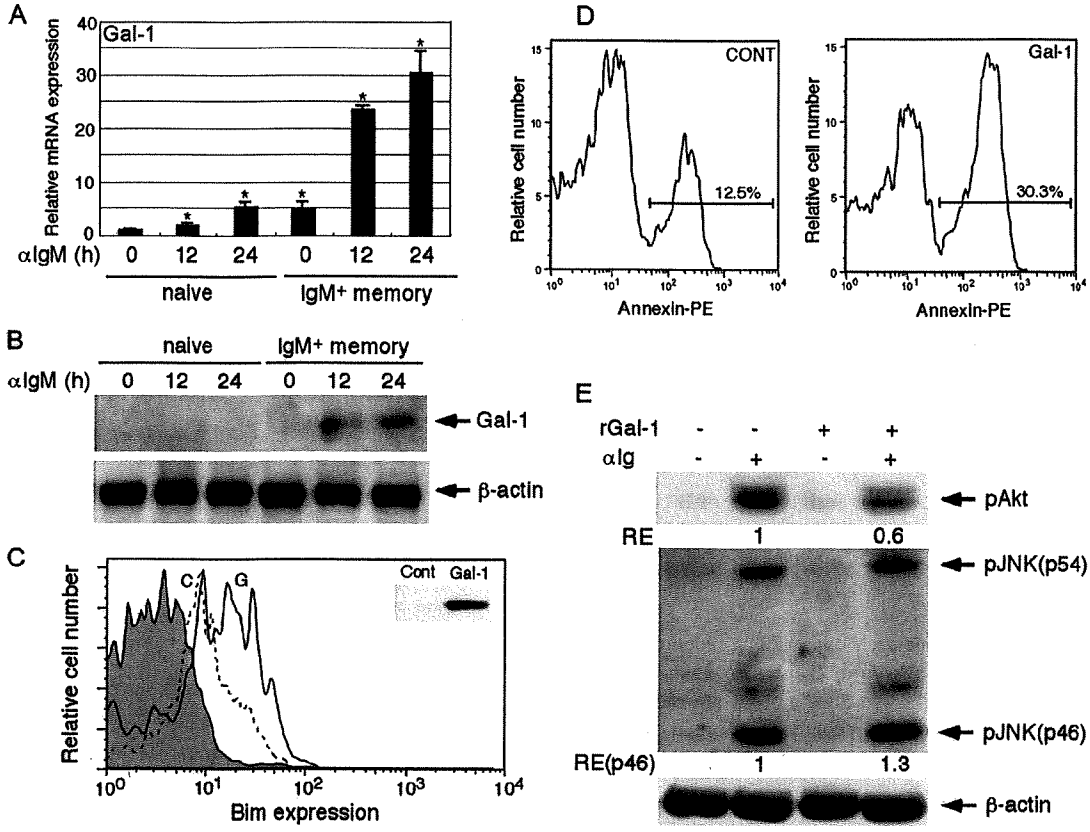

FIGURE 7. Gal-1 is critical for Bim expression and apoptosis of B cell subsets. Naive and IgM^+ memory B cells were incubated for the indicated time intervals in the absence or presence of 20 μg/ml $F(ab')_2$ goat anti (α)-hIgM. *A*, Quantitation of Gal-1 mRNA by real-time PCR in naive and IgM^+ memory B cells. Data are normalized to the expression of 18S rRNA. The results shown are representative of four independent experiments; *, $p < 0.01$ (with reference to unstimulated naive B cells). *B*, Cell lysates were separated on a 12.5% SDS-PAGE gel, and analyzed by Western blotting with anti-Gal-1 sera, and anti-β-actin mAb. Results are representative of three independent experiments. Naive B cells were transfected with either pEGFP-empty or -Gal-1 for 18 h and then stimulated with 20 μg/ml $F(ab')_2$ goat anti-human IgM for 24 h. *C*, After culture, intracellular Bim expression of GFP-positive cells was analyzed by flow cytometry. The insets depict the expression of *Gal-1* transgene. Results are representative of three independent experiments. Cont, Control. *D*, After culture, GFP-positive cells were stained with PE-labeled annexin V and analyzed using flow cytometry. Percentages of annexin-positive cells are shown. Results are representative histograms of three independent experiments. *E*, Human B cells ($CD19^+$) were pretreated with or without recombinant Gal-1 (10 μg/ml) for 12 h and stimulated for 5 min in the absence or presence of 20 μg/ml $F(ab')_2$ goat anti-human IgG/IgA/IgM. Cell lysates were separated on a 10% SDS-PAGE gel, and analyzed by Western blotting with anti-phospho-Akt, -JNK sera, and anti-β-actin mAb. The results shown are representative of three independent experiments. RE, Relative expression.

Discussion

Our study shows that BCR stimulation rescued naive B cells from apoptosis with Mcl-1 induction, whereas it rather accelerated apoptosis of IgM^+ memory B cells with Bim induction. Sustained Akt activation in naive but not IgM^+ memory B cells appears to be critical for reciprocal expression pattern of these Bcl-2 family proteins. Tcl1 and Gal-1, abundantly expressed in naive and IgM^+ memory B cells, respectively, play a crucial role in regulating Akt activation, thereby affecting their survival and death via the Bcl-2-regulated pathway.

BCR signals regulated Mcl-1 expression primarily at the protein level (Fig. 4*B*), presumably because Akt up-regulates Mcl-1 post-transcriptionally via regulating activation of glycogen synthase kinase-3 (25). Sustained Akt activation in naive B cells (Fig. 5, *B* and *C*) may thus be indispensable for continuous replenishment of Mcl-1 protein due to extraordinary short half-life of Mcl-1 (26). In contrast to Mcl-1, Bim transcription is negatively regulated by Akt through via regulating activation of the forkhead transcription factor FOXO3a (27). A small but significant increase in Bim mRNA in IgM^+ memory B cells (Fig. 4*A*) in response to BCR stimulation might be induced by immediate inactivation of Akt (Fig. 5, *B* and *C*) in this subset. Thus, Akt signals might play a critical role in controlling Mcl-1 and Bim expression reciprocally in these B cell subsets.

In contrast to BCR-induced death, spontaneous cell death is more pronounced in naive than in IgM^+ memory B cells (Fig. 3*A*). In addition, 2-day culture of naive B cells without stimuli caused a further decrease in mitochondrial membrane potential (Fig. 3*C*), suggesting that spontaneous cell death is regulated at the mitochondrial level presumably by Bcl-2 family proteins. We, however, found that in the absence of stimuli, expression levels of Bim and Mcl-1 in naive and IgM^+ memory B cells are comparable (data not shown). Therefore, Bim-Mcl-1 balances are not the main determinant of spontaneous cell death in two subsets. Collectively, Bim-Mcl-1 balances can regulate activation-induced death of B cell subsets, whereas other Bcl-2 family proteins might be more critical for the longevity of B cell subsets in the periphery.

We show here that Tcl1 and Gal-1 are differentially expressed in human naive and IgM^+ memory B cells. Tcl1 interacts with Akt and functions as a potent Akt coactivator (20, 21). In Tcl1-deficient mice, the number of splenic follicular, germinal center, and MZ B cells is reduced (28). Our data suggest that Tcl1 positively

regulates Akt activation, resulting in Mcl-1 expression in B cells (Fig. 6). To date, three Tcl1 isoforms have been identified in mice and humans: Tcl1, TCL1B, and MTCP1. Our analysis showed that Tcl1 and MTCP1 but not TCL1B mRNA are expressed in human naive and IgM^+ memory B cells, whereas the expression level of Tcl1 mRNA is different between the subsets (data not shown). These data suggest that the difference in Tcl1 expression between the subsets (Fig. 6) does not reflect the expression patterns of Tcl1 isoforms in each subset. In contrast to Tcl1, Gal-1 induced Bim protein and enhances apoptosis in B cells (Fig. 7, *C* and *D*). Furthermore, Gal-1 significantly inhibited BCR-dependent activation of Akt, leading to the up-regulation of proapoptotic Bim (Fig. 7, *C* and *E*). Gal-1 slightly enhanced BCR-induced JNK phosphorylation (Fig. 7*E*). Because JNK activation positively regulates Bim-induced apoptosis (24, 29), Gal-1 may induce Bim expression in IgM^+ memory B cells also by positively regulating JNK activation.

Gal-1 may play a critical role in the maintenance of B cell tolerance. In fact, anergic B cells express higher levels of Gal-1 than wild-type cells do (30). Gal-1 induces tolerogenic dendritic cells and promotes the expansion of regulatory T cells in vivo (31). In addition, a high level of Gal-1 is required for naturally occurring $CD4^+CD25^+$ T cells to maintain their optimal T_{reg} function (32). These data raise an interesting possibility that human IgM^+ memory B cells play a critical role in the regulation of DC and Treg functions through Gal-1 production. In contrast, abnormal expression of Tcl1 could link to the pathogenesis of B cell malignancies. Tcl1-transgenic mice reveal an expansion of the $CD5^+IgM^+$ population that is reminiscent of human B cell chronic lymphocytic leukemia (CLL) (33), and high Tcl1 expression in human B cell CLL correlates with an aggressive CLL phenotype showing unmutated Ig variable region genes and ZAP70 positivity (34). These data collectively suggest that fine-tuning of the balance between Gal-1 and Tcl1 expression is critical for the homeostasis of human B cell subsets.

Random generation of BCRs results in the emergence of a large number of self-reactive B cells, together with pathogen-specific B cells. BCR-induced cell death and anergy are thus critical for purging or silencing self-reactive B cells. However, there are significant differences in self-reactivity between human B cell subsets: in healthy individuals; up to 20% of mature naive B cells express self-reactive BCRs, whereas IgM^+ memory B cells are devoid of such self-reactive BCRs (5, 6). Bim plays a critical role in BCR-induced cell death and anergy based on the fact that Bim deficiency causes a substantial expansion of autoreactive B cells leading to autoimmune diseases (10, 35). Thus, Bim expression in IgM^+ memory B cells may serve a novel safeguard mechanism that allows efficient elimination or inactivation of the self-reactive repertoire. Our data suggest that the balance between Mcl-1 and Bim is critical in determining B cell survival and death. It has been shown that constitutive expression of B cell-activating factor of the TNF family (BAFF), a survival-promoting cytokine for murine B cells, can break B cell tolerance through expanding self-reactive B cell populations in MZ (36, 37). BAFF exerts its effects on murine B cell survival through down-regulating Bim and up-regulating Mcl-1 (38, 39). We found that BCR-induced death in human IgM^+ memory B cells is abrogated in the presence of BAFF (data not shown). Because patients with systemic lupus erythematosus and Sjögren's syndrome have elevated levels of serum BAFF (37), it is important to test whether self-reactive IgM^+ memory B cells are expanded in these autoimmune diseases.

In summary, BCR signaling dictates survival and death in human naive and IgM^+ memory B cells, respectively. These phenotypes are driven by reciprocal expression of Bcl-2 family proteins such as Mcl-1 and Bim in these B cell subsets. Tcl1 and Gal-1 are expressed in naive and IgM^+ memory B cell subsets, respectively. Tcl1 and Gal-1 might play critical roles in the expression of Mcl-1 and Bim, at least through regulating Akt activation. Therefore, a unique set of molecules such as Tcl1 and Gal-1 defines distinct BCR signaling cascades, dictating fate of human naive and IgM^+ memory B cells.

Disclosures

The authors have no financial conflict of interest.

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ARTICLE

Molecular explanation for the contradiction between systemic Th17 defect and localized bacterial infection in hyper-IgE syndrome

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Hyper-IgE syndrome (HIES) is a primary immunodeficiency characterized by atopic manifestations and susceptibility to infections with extracellular pathogens, typically *Staphylococcus aureus*, which preferentially affect the skin and lung. Previous studies reported the defective differentiation of T helper 17 (Th17) cells in HIES patients caused by hypomorphic *STAT3* mutations. However, the apparent contradiction between the systemic Th17 deficiency and the skin/lung-restricted susceptibility to staphylococcal infections remains puzzling. We present a possible molecular explanation for this enigmatic contradiction. HIES T cells showed impaired production of Th17 cytokines but normal production of classical proinflammatory cytokines including interleukin 1β. Normal human keratinocytes and bronchial epithelial cells were deeply dependent on the synergistic action of Th17 cytokines and classical proinflammatory cytokines for their production of antistaphylococcal factors, including neutrophil-recruiting chemokines and antimicrobial peptides. In contrast, other cell types were efficiently stimulated with the classical proinflammatory cytokines alone to produce such factors. Accordingly, keratinocytes and bronchial epithelial cells, unlike other cell types, failed to produce antistaphylococcal factors in response to HIES T cell–derived cytokines. These results appear to explain, at least in part, why HIES patients suffer from recurrent staphylococcal infections confined to the skin and lung in contrast to more systemic infections in neutrophil-deficient patients.

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Abbreviations used: BD, β-defensin; CAA, *Candida albicans* antigen; CGD, chronic granulomatous disease; HIES, hyper-IgE syndrome; HMVEC-L, human lung microvascular endothelial cell; HUVEC, human umbilical vein endothelial cell; SEB, staphylococcal enterotoxin B; TLR, Toll-like receptor.

The identification of Th17 cells as a third subset of helper T cells has illuminated the fact that distinct subsets of helper T cells have been evolved to protect our body from infections by various types of microorganisms and are involved differently in the induction and exacerbation of various immunological disorders. Th17 cells are characterized and distinguished from IFN-γ–producing Th1 cells and IL-4–producing Th2 cells by their production of so-called Th17 cytokines including IL-17 (IL-17A), IL-17F, and IL-22 (1–4). For their differentiation from naive CD4 T cells, Th17 cells require different cytokines and transcription factors than do Th1, Th2, or regulatory T cells. The roles of Th17 cells in immune responses are also different from those of other helper T cells. In particular, their pathological roles in autoimmune and inflammatory diseases, including multiple sclerosis, rheumatoid arthritis, psoriasis, and inflammatory bowel diseases, have been studied extensively (5–10).

Although the functions of Th17 cells under physiological conditions have not been completely elucidated, accumulating data suggest that Th17 cells play crucial roles in the host defense against extracellular pathogens that are

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not efficiently cleared by Th1- and Th2-type immune responses. Th17-type cytokines IL-17A and IL-17F are important for the recruitment of neutrophils (11), whereas IL-22 induces the production of antimicrobial peptides β-defensin (BD) 2 and BD3 by keratinocytes, through the activation of STAT3 (12–14). Mice with a homozygous deletion of the gene encoding the IL-17RA (IL-17 receptor A) and mice that do not produce IL-22 are susceptible to lung infection by the Gram-negative bacteria *Klebsiella pneumoniae* and *Mycoplasm pulmonis* (15–17). Mice that produce neither IL-17A nor IL-17F are susceptible to skin infection by the Gram-positive bacteria *Staphylococcus aureus* (18). Administration of anti–IL-17A neutralizing antibodies impairs both the intra-abdominal abscess formation in response to *Bacteroides fragilis* and *Escherichia coli* (19–21) and the host defense against systemic infection by the fungus *Candida albicans* (22). These data indicate that Th17 cells play a key role in immune responses to extracellular bacteria and fungi in mice. In contrast, the anti-pathogenic roles of Th17 cells in humans are relatively uncertain.

Recent studies demonstrated that the differentiation of human Th17 cells was defective in patients with hyper-IgE syndrome (HIES) (23–26). HIES is a primary immunodeficiency disease caused by dominant-negative mutations in the DNA-binding domain, SH2 domain, or transactivating domain of STAT3 (26–28). As expected from the important roles of STAT3 in transducing signals for a variety of cytokines, growth factors, and hormones, patients with HIES display complex clinical manifestations in multiple organs, including atopic dermatitis with high serum IgE levels and abnormalities of the bones and teeth (29–32). Most patients suffer from recurrent infections by fungi and bacteria, predominantly the Gram-positive bacteria *S. aureus*. The presence of these infections suggests that Th17 cells play a crucial role in protection from extracellular pathogens, not only in mice but also in humans. However, curiously, the staphylococcal infections in HIES patients are often confined to the skin and lung and manifest clinically as skin abscesses and cyst-forming pneumonia. These skin- and lung-restricted infections are in sharp contrast to the pattern of infection observed in patients with a neutrophil deficiency. For example, in patients with chronic granulomatous disease (CGD), staphylococcal infections occur in a wide variety of organs including the lung, lymph nodes, skin, liver, bone, gastrointestinal tract, kidney, and brain (33). Thus, it remains elusive why HIES patients suffer from skin- and lung-restricted staphylococcal infections in spite of their systemic Th17 deficiency.

In the present study, we explored possible molecular mechanisms underlying the recurrent staphylococcal infections confined to the skin and lung in HIES patients. We found that primary human keratinocytes and bronchial epithelial cells displayed a much stronger dependence than other cell types on Th17 cytokines in their production of antistaphylococcal factors including the neutrophil-recruiting chemokines and antibacterial peptides. T cells from HIES patients, in spite of their defect in production of Th17 cytokines, showed normal production of other proinflammatory cytokines, including IL-1β, which was insufficient for triggering keratinocytes and bronchial epithelial cells but sufficient for other cell types to produce antistaphylococcal factors. Th17 cytokines and classical proinflammatory cytokines synergistically stimulated keratinocytes and bronchial epithelial cells, but the synergy was not seen in other types of cells. These findings provide a possible molecular explanation for the apparent contradiction between the systemic Th17 deficiency and the skin and lung-restricted staphylococcal infections in HIES patients.

RESULTS

HIES T cells produce little or no Th17 cytokines and fail to stimulate keratinocytes to secrete neutrophil-recruiting chemokines and BDs

We first examined the profile of cytokines produced by T cells from our cohort of HIES patients whose *STAT3* genes carried mutations. The amounts of IL-17A and IL-22 secreted from the patients' T cells upon stimulation with anti-CD3 and anti-CD28 mAbs were invariably only ~5–10% of those from healthy control subjects, which is in accordance with previous results (23–26), whereas the production levels of IFN-γ, IL-1β, and TNF-α were comparable in the two groups (Fig. 1). Real-time quantitative RT-PCR demonstrated that the up-regulation of *IL-17F* expression by the patients' T cells was also impaired (unpublished data). Thus, the patients' T cells showed a selective defect in the production of Th17 cytokines.

We next investigated the functional consequences of the Th17 deficiency in the context of staphylococcal infections of the skin. Normal human primary epidermal keratinocytes were cultured in vitro with culture supernatants from HIES patients' or control subjects' T cells that had been unstimulated or stimulated with anti-CD3 plus anti-CD28. The expression and production of two chemokines, CXCL8 (IL-8) and CCL2, was up-regulated in the keratinocytes cultured with the conditioned medium from activated control T cells (Fig. 2 A and Fig. S1). In contrast, although CCL2 was also up-regulated by the conditioned medium from activated HIES T cells, CXCL8 was not (Fig. 2 A and Fig. S1). Among the three antimicrobial peptides (BDs) examined, at the mRNA and protein level the expression of BD1 but not BD2 or BD3 was up-regulated in keratinocytes when they were stimulated with conditioned medium from the T cells of HIES patients, but all three were up-regulated by the conditioned medium from the control subjects' T cells (Fig. 2 A and Fig. S1). Thus, the HIES patients' T cells could not stimulate keratinocytes to produce a significant amount of the neutrophil-recruiting chemokine CXCL8 or the antimicrobial peptides BD2 and BD3, but they could stimulate the up-regulation of CCL2 and BD1.

T cell–derived Th17 cytokines are responsible for the production of CXCL8 and BDs from keratinocytes

When the supernatants from activated control T cells were treated with the combination of anti–IL-17A and anti–IL-22 blocking mAbs before incubation with keratinocytes, their capability of stimulating keratinocytes to up-regulate CXCL8, BD2, and BD3 was diminished to the level displayed by the

HIES patients' T cells (Fig. 2 A and Fig. S1). Either anti–IL-17A or anti–IL-22 alone was less effective than their combination (Fig. S2). Thus, the defective production of chemokines and BDs by keratinocytes in response to the conditioned medium from the patients' T cells was attributable to the T cells' defective production of Th17 cytokines.

We next examined the direct effect of the keratinocyte-derived factors on the growth of *Staphylococcus aureus* using a colony-forming assay (Fig. 2, B and C). When bacteria were cultured with the culture supernatant from keratinocytes stimulated by control T cells, the number of bacterial colonies was reduced to 60% as compared with that when cultured with control medium. However, this was not the case when HIES T cells were used to stimulate keratinocytes. The antibacterial activity was completely abrogated when the culture supernatant from the control T cells was pretreated with the blocking mAbs for IL-17A and IL-22 before it was added to the keratinocytes (Fig. 2 B), and it was attenuated when the keratinocyte supernatants were pretreated with an anti-BD3 mAb before their application to the bacterial culture (Fig. 2 C). These results indicated that control but not HIES T cells produced Th17 cytokines that, in turn, acted on keratinocytes to elicit their secretion of antimicrobial factors including BD3.

Keratinocytes and bronchial epithelial cells display a greater dependence on Th17 cytokines for their production of chemokines and BDs than other cell types

To learn why the staphylococcal infections are confined to the skin and lung in HIES patients, we analyzed different lineages of human primary cells for their ability to secrete chemokines and BDs in response to T cell–derived factors including Th17 cytokines. We first compared their responsiveness to culture supernatants from either control or patient T cells that were activated with anti-CD3 and anti-CD28 mAbs. Primary bronchial epithelial cells responded to the T cell conditioned medium just as the primary keratinocytes did. That is, both cell types up-regulated the expression and production of chemokines (CXCL8 and CXCL1) and BDs (BD2 and BD3) when incubated with the supernatants from control T cells but not from HIES T cells (Fig. 3 A and Fig. S3). Interestingly, primary dermal fibroblasts, human umbilical vein endothelial cells (HUVEC), and human lung microvascular endothelial cells (HMVEC-L) responded equally well, in terms of their secretion of the chemokines and BDs, to the supernatants from control or patient T cells (Fig. 3 A and Fig. S3). This was also true for the expression and production of CXCL8 and CXCL1 by human macrophages (Fig. 3 A and Fig. S3). Human macrophages did not produce detectable amounts of BDs. Thus, keratinocytes and bronchial epithelial cells responded differently to T cell–derived factors than the other cell types tested and appeared to be much more dependent on Th17 cytokines for their induction to secrete chemokines and BDs.

These findings prompted us to examine the responses of different cell types to individual cytokines and their combinations, including the Th17 cytokines (IL-17A, IL-17F, and IL-22), classical proinflammatory cytokines (IL-1β, TNF-α, and IFN-γ), or both. Keratinocytes secreted CXCL8 in response to IL-17A, IL-22, IL-1β, or TNF-α in a dose-dependent

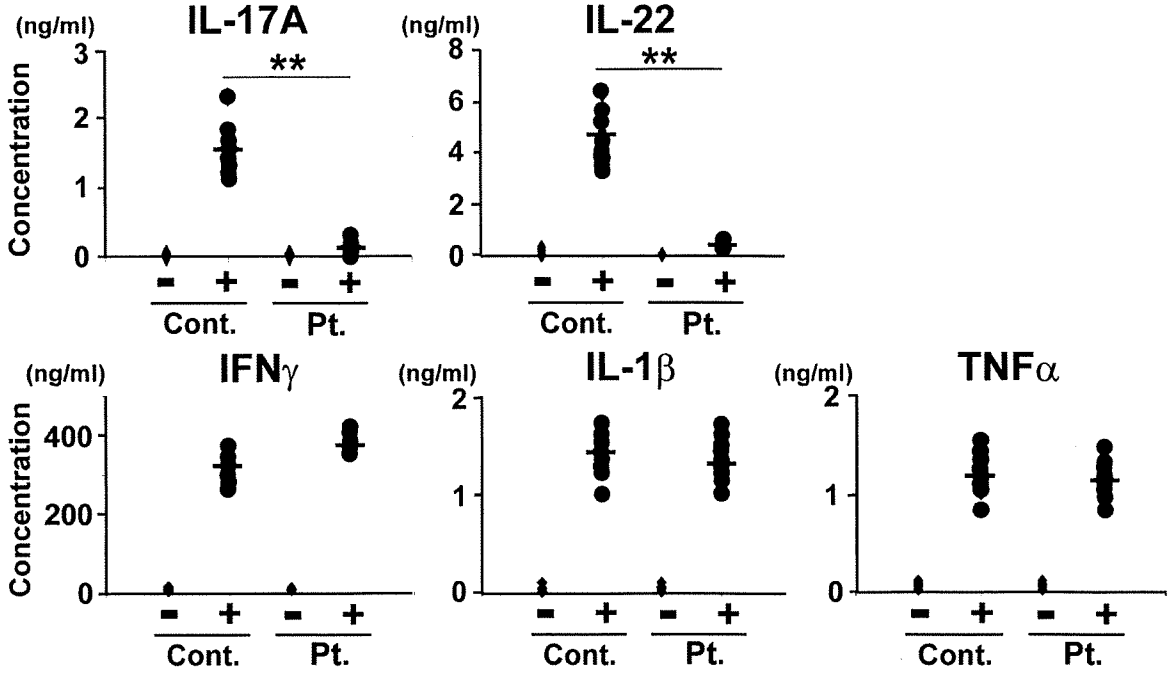

Figure 1. HIES T cells produce greatly reduced amounts of Th17 cytokines and normal amounts of classical proinflammatory cytokines upon activation. PBMCs from HIES patients (Pt.) and control subjects (Cont.; $n = 8$ each, indicated by dots) were stimulated (+) or not (−) with anti-CD3 and anti-CD28 for 72 h, and the concentration of the indicated cytokines in their culture supernatants was determined by ELISA. The results shown are representative of three independent experiments. **, $P < 0.01$.

manner, but they responded poorly to IL-17F and IFN-γ (Fig. S4 A). Using the Th17 cytokine cocktail or the classical proinflammatory cytokine cocktail resulted in some additive effect on the keratinocytes' secretion of CXCL8 (Fig. S4, B and C). In contrast, the combination of both types of cytokines dramatically enhanced the CXCL8 production by the keratinocytes (Fig. 3 B and Fig. S5). This was also the case for bronchial epithelial cells (Fig. 3 B). In contrast, fibroblasts, HUVEC, and HMVEC-L secreted a large quantity of CXCL8 in response to the classical proinflammatory cytokine cocktail, but the further addition of Th17 cytokine cocktail caused no significant enhancement of CXCL8 production (Fig. 3 B). Furthermore, the Th17 cytokine cocktail was much less effective in stimulating fibroblasts, HUVEC, and HMVEC-L than the classical cytokine cocktail, and the amount of CXCL8 produced by the Th17 cytokine cocktail-treated fibroblasts, HUVEC, and HMVEC-L was ~10–30% of that produced by stimulation with the classical proinflammatory cocktail. Macrophages responded to the cytokines in a pattern similar to fibroblasts, HUVEC, and HMVEC-L, although the macrophages produced 10× less CXCL8 than the others.

In keratinocytes and bronchial epithelial cells, the marked synergy caused by combining the Th17 and classical proinflammatory cytokines affected not only the expression of CXCL8 but also that of other chemokines (CXCL1 and CXCL2) and BDs (BD2 and BD3; Fig. S6 A). In accordance with this finding, the supernatants of keratinocytes stimulated with the Th17–classical cytokine combination caused robust neutrophil chemotaxis compared with the supernatants from keratinocytes stimulated with only one of the cocktails (Fig. S6 B).

Previous studies demonstrated that the stimulation of keratinocytes with toll-like receptor (TLR) 2 ligands induces the production of the chemokines and antimicrobial peptides (34, 35). In agreement with this, the keratinocytes showed up-regulated CXCL8 secretion and BD expression in response to lipoteichoic acid, peptidoglycan, or fixed *S. aureus*, but the extent of up-regulation was <10% of that observed after stimulation with the combination of Th17 and proinflammatory cytokines (Fig. S6 C).

Molecular mechanisms underlying the unique responsiveness of keratinocytes and bronchial epithelial cells to Th17 cytokines in synergy with other proinflammatory cytokines

To explore the possible molecular basis of the poorer response of keratinocytes and bronchial epithelial cells to the

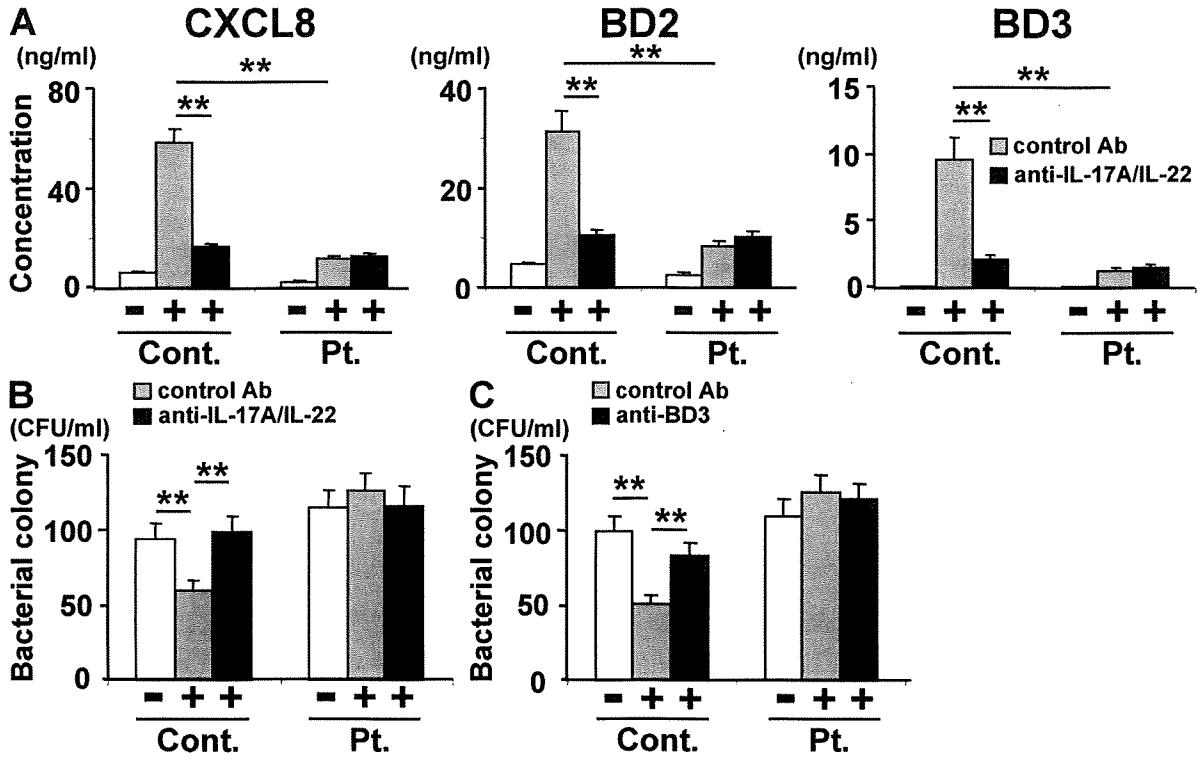

Figure 2. Supernatants of activated HIES T cells fail to stimulate keratinocytes to secrete significant amounts of antibacterial factors. In the presence or absence of anti–IL-17A plus anti–IL-22 (A and B), anti-BD3 (C), or isotype-matched control antibodies, primary human keratinocytes were incubated for 48 h with the supernatants of HIES (Pt.) or control (Cont.) T cells that had been stimulated (+) or not (−) with anti-CD3 and anti-CD28 for 72 h as in Fig. 1. (A) The concentration of CXCL8, BD2, and BD3 in keratinocytes supernatants was determined by ELISA. Representative data from one patient and one control are shown (mean ± SD; $n = 3$), and similar results were obtained from the other patients and controls. (B and C) The culture supernatants of keratinocytes were analyzed for their antistaphylococcal activity by the colony assay (mean ± SD; $n = 3$). The results shown in are representative of at least three independent experiments. **, $P < 0.01$.

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classical proinflammatory cytokines, as compared with the other types of cells, we analyzed the expression of the classical cytokine IL-1R1 (IL-1 receptor) and its antagonists IL-1R2 and IL-1Ra (Fig. 4, A and B). Compared with fibroblasts, keratinocytes expressed 1/600th of IL-1R1 transcripts, 170-fold more IL-1Ra transcripts, and 260-fold more IL-1R2 (Fig. 4 A). The great difference in their expression between keratinocytes and fibroblasts was also confirmed at the protein level (Fig. 4 B). Bronchial epithelial cells showed the keratinocyte-type expression, whereas HUVEC and HMVEC-L displayed the fibroblast-type expression (Fig. 4 A and not depicted). Consistent with this result, keratinocytes showed a much poorer up-regulation of c-Fos and IL-6 than fibroblasts in response to IL-1β (Fig. 4 C). This appeared to partly explain why keratinocytes and bronchial epithelial cells were less sensitive to the classical proinflammatory cytokines than fibroblasts but did not account for the strong synergy between the Th17 and the classical proinflammatory cytokines in the keratinocytes. Therefore, we next examined the possible cross-talk between the two types of cytokines in terms of the regulation of cytokine receptor expression.

We found that the expression of the Th17 cytokine receptors IL-17RA, IL-17RC, and IL-22R was up-regulate, in keratinocytes incubated with the classical proinflammatory

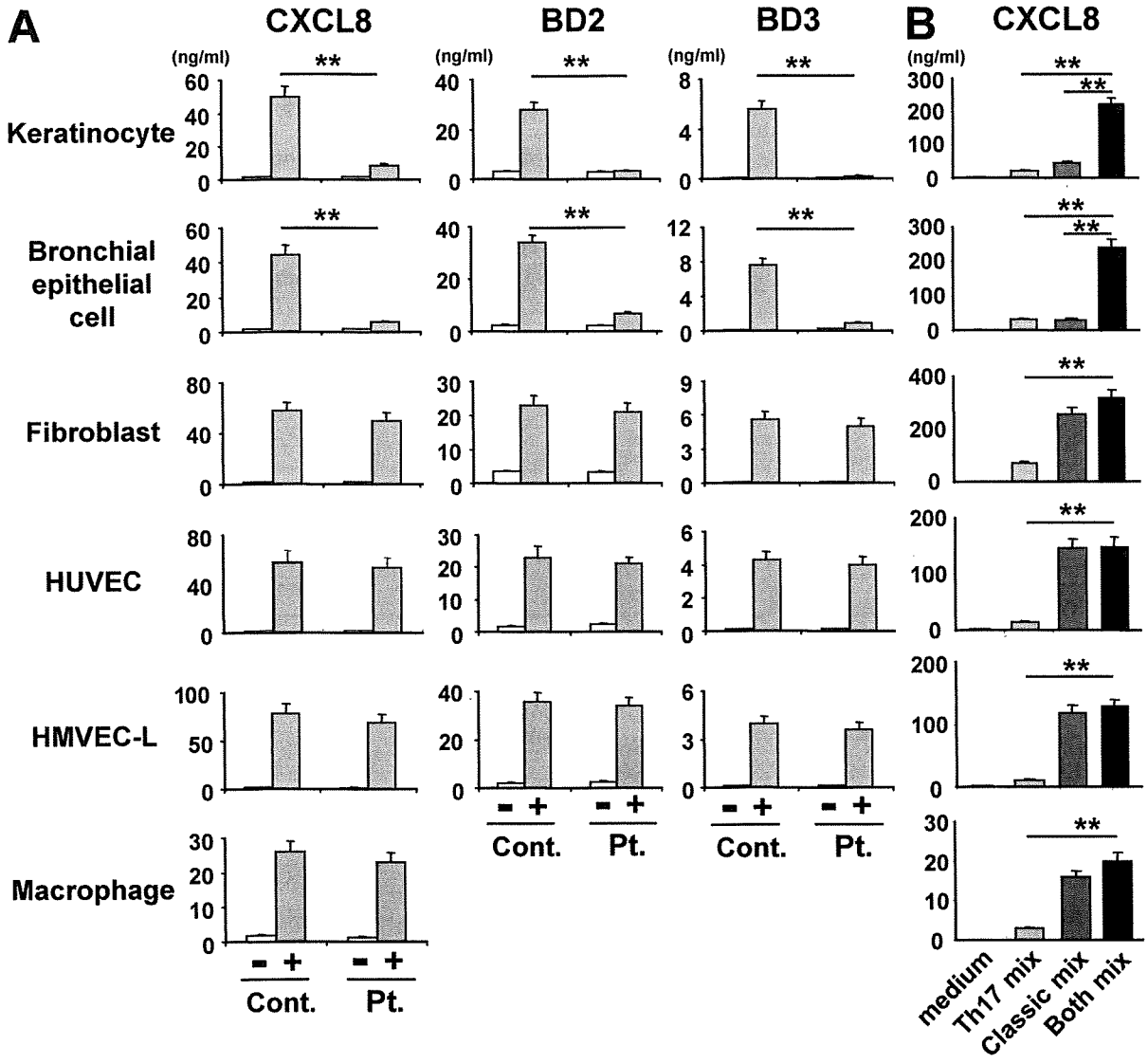

Figure 3. Keratinocytes and bronchial epithelial cells show greater dependence on Th17 cytokines for the production of chemokines and BDs than other cell types. Primary human keratinocytes, bronchial epithelial cells, dermal fibroblasts, endothelial cells (HUVEC and HMVEC-L), and macrophages were incubated for 48 h with T cell supernatants that were prepared as described in Fig. 1 A or with the Th17 cytokine cocktail (Th17 mix: IL-17A + IL-17F + IL-22), the classical proinflammatory cytokine cocktail (classical mix: TNF-α + IL-1β + IFN-γ), or the combination of both (both mix; B). The concentration of CXCL8, BD2, and BD3 in their culture supernatants was determined by ELISA. Representative data from one patient and one control are shown in A (mean ± SD; $n = 3$), and similar results were obtained from the other patients and controls. The results shown are representative of at least three independent experiments. **, $P < 0.01$.

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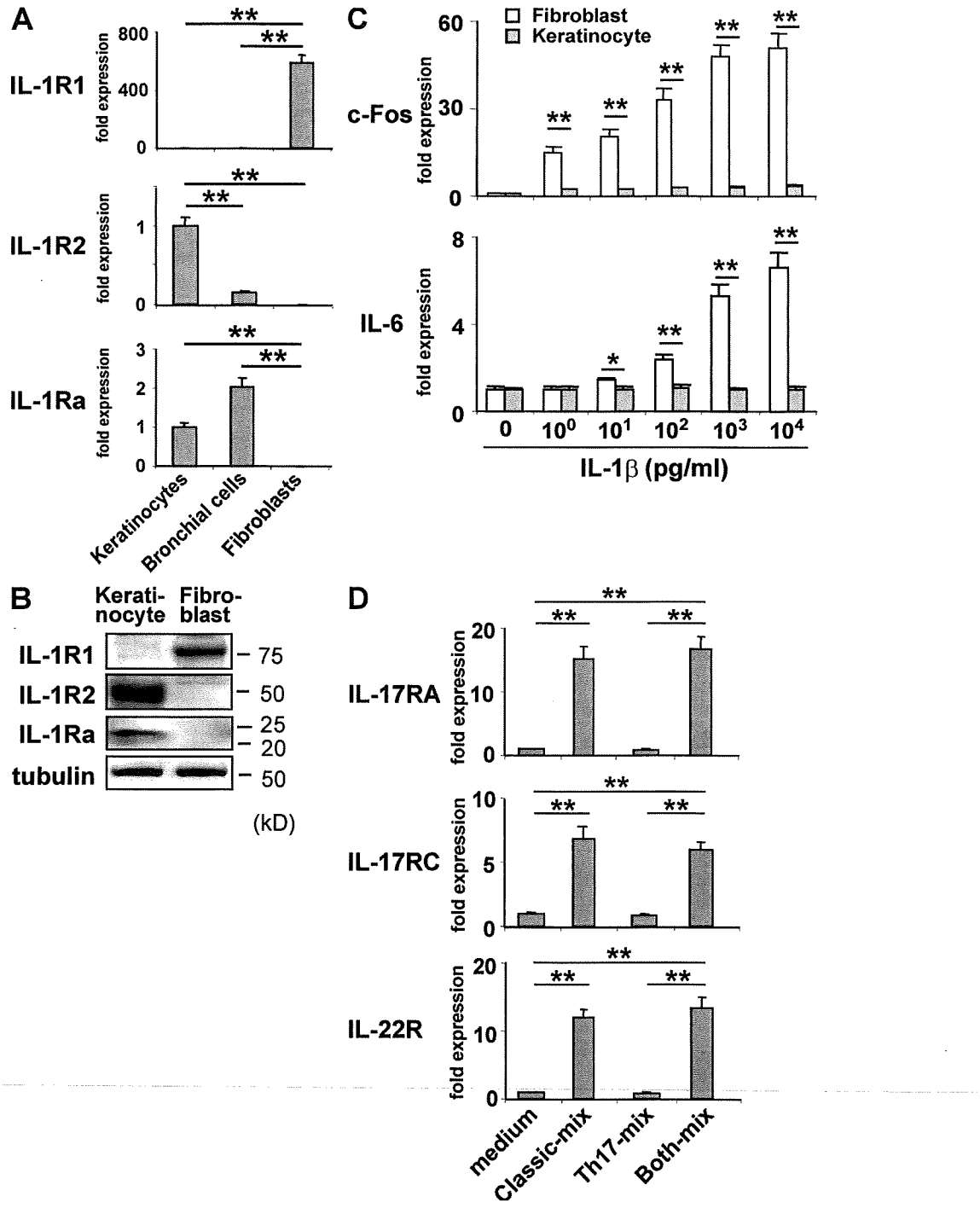

Figure 4. Distinct expression and regulation of the cytokine receptors in keratinocytes and fibroblasts compared with those in other cell types. (A) The expression of *IL-1R1*, *IL-1Ra*, and *IL-1R2* in the indicated cells was determined by quantitative RT-PCR. Data shown were normalized to *HPRT* levels, and the level of expression in keratinocytes was defined as 1.0. (B) IL-1R1, IL-1R2, and IL-1Ra proteins in keratinocytes and fibroblasts were detected by immunoblotting. (C) Keratinocytes and fibroblasts were cultured for 15 min with the indicated concentration of IL-1β and analyzed by quantitative RT-PCR for the expression of *c-Fos* and *IL-6*. Data shown were normalized to HPRT levels, and the level of expression in cells cultured without IL-1β was defined as 1.0 for each cell type. (D) Keratinocytes cultured as in Fig. 3 B were analyzed by quantitative RT-PCR for the expression of *IL-17RA*, *IL-17RC*, and *IL-22R*. The data shown were normalized to the *HPRT* levels, and the level of expression in cells cultured without any added cytokine was defined as 1.0. The results shown are representative of three independent experiments. Error bars show mean ± SD ($n = 3$). *, $P < 0.05$; **, $P < 0.01$.

cytokines but not the Th17 cytokines (Fig. 4 D and Fig. S7). Conversely, in keratinocytes incubated with the Th17 cytokines, the expression of the receptors for the classical proinflammatory cytokines was up-regulated, albeit less markedly (unpublished data). This reciprocal up-regulation of cytokine receptor expression was also observed in bronchial epithelial cells (unpublished data). These findings could account, at least in part, for the synergistic effect of the Th17 and the classical proinflammatory cytokines on the production of antibacterial factors by keratinocytes and bronchial epithelial cells.

HIES T cells show poor ability of stimulating keratinocytes in response to staphylococcal superantigens and candida antigens

We next investigated the responses of HIES T cells under more clinically relevant conditions to obtain a better insight into the susceptibility to staphylococcal infections observed in HIES patients. When stimulated with the *S. aureus*–derived superantigens for T cells, staphylococcal enterotoxin B (SEB), HIES patients' T cells produced drastically reduced amounts of IL-17A and IL-22, <10% of those produced by control T cells (Fig. 5 A). In contrast, the IL-1β production was normal, and the IFN-γ and TNF-α production was even enhanced in SEB-stimulated HIES T cells (Fig. 5 A). It is of note that the supernatants of SEB-stimulated HIES T cells showed much poorer ability to induce the production of CXCL8 and BD2 in keratinocytes compared with those from control T cells (Fig. 5 B). In contrast, both supernatants from HIES and control T cells almost equally well stimulated fibroblasts to produce CXCL8 and BD2. The combination of anti–IL-17A and –IL-22 efficiently inhibited the CXCL8/BD2-inducing activity of control T cells' supernatants in keratinocytes but showed no significant inhibition in the CXCL8/BD2 production from fibroblasts that were stimulated with the supernatants from either control or HIES T cells (Fig. 5 B). These results strongly suggested that Th17

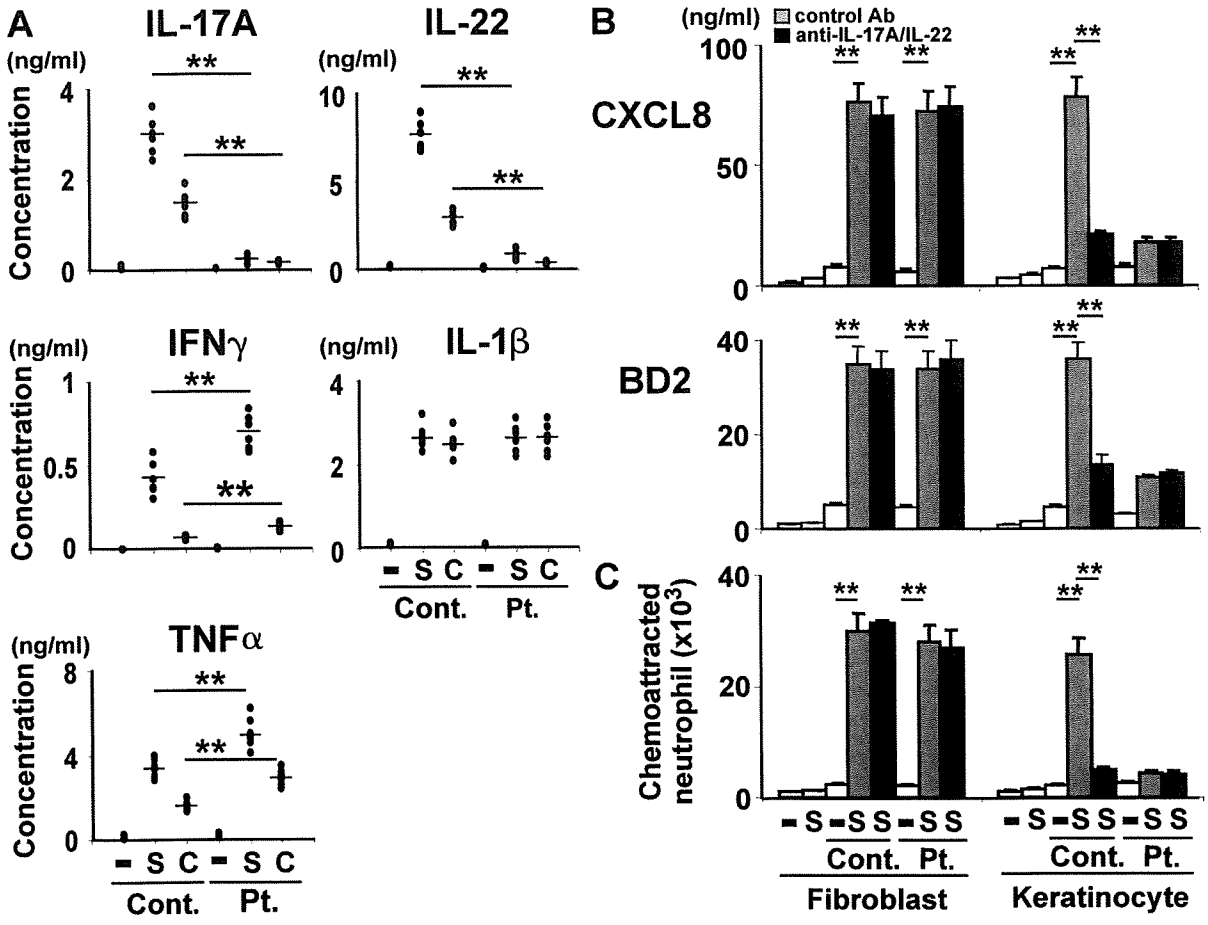

Figure 5. HIES T cells show poor ability of stimulating keratinocytes in response to staphylococcal superantigens and candida antigens. (A) PBMCs from HIES patients (Pt.) and control subjects (Cont.; $n = 6$ each, indicated by dots) were stimulated or not (−) with SEB (S, 100 ng/ml) or CAA (C, 1/20,000 vol/vol) for 5 d, and the concentration of the indicated cytokines in their culture supernatant was determined by ELISA. (B and C) Fibroblasts and keratinocytes were cultured for 48 h in the absence (−) or presence (S) of SEB or with the supernatants of patients (Pt.) or control (Cont.) PBMCs that had been unstimulated (−) or stimulated with SEB (S) as in A, in the presence or absence of anti–IL-17A + anti–IL-22 or isotype-matched control antibodies. Their culture supernatants were analyzed by ELISA for the secretion of CXCL8 and BD2 (B) and evaluated for their neutrophil chemotactic activity (C). The results shown are representative of two independent experiments. Error bars show mean ± SD ($n = 3$). **, $P < 0.01$.

cytokines secreted by SEB-stimulated T cells played a critical role in the induction of CXCL8 and BD2 production in keratinocytes but not in fibroblasts. In accord with these results, supernatants of keratinocytes stimulated with HIES T cells showed little or no ability of neutrophil chemoattraction, whereas those of keratinocytes stimulated with control T cells and those of fibroblasts stimulated with either control or HIES T cells induced robust neutrophil chemotaxis (Fig. 5 C). These in vitro findings may account in part, if not entirely, for the skin/lung-confined susceptibility to staphylococcal infections observed in HIES patients.

We further examined the responsiveness of HIES T cells to *Candida albicans* antigen (CAA). HIES T cells showed impaired cytokine production in response to CAA in the essentially same pattern as observed in response to SEB (Fig. 5 A). This may also explain in part the incidence of mucocutaneous infections with *C. albicans* that is often observed in HIES patients.

DISCUSSION

In the present study, we demonstrated that skin and lung epithelial cells displayed an unusual pattern of responsiveness to Th17 and other proinflammatory cytokines that was distinct from that of the other cell types tested. This previously unrecognized modes of cytokine responses could fill in the apparent gap between the systemic Th17 deficiency and the tissue-dependent susceptibility to staphylococcal infections in the HIES patients. Both Th17 cytokines and other classical proinflammatory cytokines stimulate a variety of cells to produce neutrophil-recruiting chemokines and antimicrobial peptides, which are important for providing protection against bacterial infections (7). We found that skin and lung epithelial cells efficiently secreted antibacterial factors only when stimulated with a combination of Th17 cytokines and classical proinflammatory cytokines. These observations were made using primary cells that were grown on plastic. In contrast, fibroblasts, endothelial cells, and macrophages efficiently secreted antibacterial factors when stimulated with the classical proinflammatory cytokines alone. Thus, skin and lung epithelial cells showed a much higher dependence on Th17 cytokines, in synergy with the classical proinflammatory cytokines, than the other cell types. The classical proinflammatory cytokines up-regulated the expression of Th17 cytokine receptors and, conversely, the Th17 cytokines up-regulated the expression of receptors for the classical proinflammatory cytokines, albeit less strongly. This reciprocal up-regulation of cytokine receptor expression could be one of the molecular mechanisms underlying the strong synergy between the Th17 and classical proinflammatory cytokines in skin and lung epithelial cells.

This synergistic action of the cytokines appears to account in part, if not entirely, for the skin- and lung-restricted staphylococcal infections of HIES patients. *S. aureus* produces enterotoxins, including SEB, that function as superantigens to stimulate the bulk of T cells. The HIES patients' T cells showed impaired production of Th17 cytokines in response to SEB but normal production of the classical proinflammatory cytokines. Therefore, the skin and lung epithelial cells of HIES patients, unlike other cell types, probably do not secrete sufficient amounts of neutrophil-recruiting chemokines and the antimicrobial peptide BDs to fend off staphylococcal infection. With regard to the sites of bacterial infections, it is important to consider the pathogen's characteristics. The tendency of *S. aureus* to colonize the skin and upper respiratory tract may explain the skin/lung-restricted staphylococcal infections observed in HIES patients. It is of note, however, that CGD patients suffer from staphylococcal infections that occur in a wide variety of organs, including the lung, lymph nodes, skin, liver, bone, gastrointestinal tract, kidney, and brain (33). The difference in the spectrum of affected tissues between HIES and CGD patients strongly suggests that the host factors, in addition to the pathogen's intrinsic factors, would determine the preferential sites of infections. In HIES patients, unlike in CGD patients, the neutrophils themselves are normal in their number and function; however, they probably cannot be recruited to the skin and lung because HIES T cells cannot induce the skin and lung epithelial cells to produce neutrophil-recruiting chemokines like CXCL8, even though we cannot formally exclude the possibility that the STAT3 mutation in the epithelial cells of HIES patients also contributes to impaired production of antistaphylococcal factors including CXCL8. Mice deficient for IL-17RA or IL-22 and mice treated with an anti–IL-17A blocking antibody are susceptible to infections with Gram-negative bacteria, such as *K. pneumoniae*, *M. pulmonis*, *B. fragilis*, *E. coli*, and *Citrobacter rodentium*, which are rarely observed in HIES patients (16, 17, 20, 21, 36, 37). The reason for this difference between human and mouse in bacterial susceptibility remains to be determined.

TLR-mediated signals are known to be important for immune protection from staphylococcal infections. The outer cell wall of *Staphylococcus aureus* is composed of exposed peptidoglycan and lipoteichoic acid, which are recognized by TLR2 (38–40). Mice deficient in TLR2 and patients deficient in IRAK4, a transducer of TLR signaling, show increased susceptibility to staphylococcal infections (41–43). Importantly, TLR2 signaling is intact in HIES patients (44, 45). We demonstrated in the present study that keratinocytes indeed produced antibacterial factors in response to TLR2 ligands, but the amounts were <10% of those induced when the cells were stimulated with the combination of Th17 and classical proinflammatory cytokines. Thus, TLR2-mediated signaling alone appears to be insufficient for the full protection against staphylococcal infection of the human skin and lung.

Skin and lung epithelial cells are located, respectively, at the major outer and inner surface barriers of the body, and are constantly exposed to agents from the environment. Therefore, these cells probably need to discriminate between infectious and noninfectious agents to avoid unnecessary inflammation. The present study demonstrated that they secrete antibacterial factors only when they receive stimuli from both classical proinflammatory cytokines delivered by innate immunity–type cells and Th17 cytokines delivered by T cells. Thus, an attractive hypothesis is that epithelial cells have been equipped by evolution to respond poorly to the first alert

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signal, i.e., the classical proinflammatory cytokines produced by innate immunity cell types, which might be evoked even by noninfectious agents. Infectious agents, such as *S. aureus*, could evoke the production of the second alert signal, i.e., Th17 cytokines produced by T cells, in addition to the first alert signal. This would allow the epithelial cells to respond selectively to pathogens. In HIES patients, the second alert signal is not delivered because of the Th17 deficiency, which probably results in skin- and lung-restricted staphylococcal infections.

Accumulating evidence indicates that Th17 cells and their products are very important in the induction and propagation of autoimmunity (5–10). Therefore, the neutralization of Th17 cytokines appears to be a promising therapeutic strategy for the control of inflammation in autoimmune disorders. Moreover, antagonists of STAT3 are considered good candidates for the treatment of tumors because a variety of tumor cells show up-regulated STAT3 expression (46). However, our data indicate that such treatments would render patients susceptible to staphylococcal infection, particularly of the skin and lung, as observed in HIES. Fortunately, our study also suggests that this undesirable side effect might be prevented or treated by the local application of neutrophil-recruiting chemokines and BDs or their derivatives.

In summary, we demonstrated in the present study that T cells from HIES patients, in spite of their defect in production of Th17 cytokines, showed normal production of other proinflammatory cytokines including IL-1β in response to staphylococcal antigens, which was insufficient for triggering keratinocytes and bronchial epithelial cells but sufficient for other cell types to produce antistaphylococcal factors. This provides a possible molecular explanation for the apparent contradiction between the systemic Th17 deficiency and the skin- and lung-restricted staphylococcal infections in HIES patients.

MATERIALS AND METHODS

Patients. All eight patients enrolled in this study had typical findings associated with HIES and a National Institutes of Health score >40 points (27). The diagnosis was confirmed by the identification of the mutations in the *STAT3* gene. The study was approved by the Tokyo Medical and Dental University Ethics Committee, and written informed consent was obtained from the patients. All of the patients were in a healthy state when their blood samples were collected. Blood samples from patients and age-matched healthy subjects were obtained and PBMCs were prepared by density-gradient centrifugation.

Cell culture. PBMCs were cultured in 96-well plates in RPMI medium 1640 supplemented with 1% penicillin/streptomycin, 1% glutamine, and 10% heat-inactivated FCS. Cultures were stimulated with a 1:100 (vol/vol) dilution of anti-CD3 and anti-CD28 beads (Invitrogen). For some experiments, the following mAbs, cytokines, and TLR ligands were added: 20 ng/ml IL-17A, 200 ng/ml IL-17F, and 200 ng/ml IL-22 (R&D Systems); 10 ng/ml TNF-α, 10 ng/ml IL-1β, 10 ng/ml IFN-γ (PeproTech); neutralizing antibodies against IL-17, IL-22, and BD3 (R&D Systems); TLR ligands (InvivoGen); fixed *S. aureus* (EMD); SEB (Toxin Technology); and *C. albicans* skin test antigen (Torii Pharmaceutical Co., Ltd).

Culture of human keratinocytes, bronchial epithelial cells, fibroblasts, endothelial cells, and macrophages. Human epidermal keratinocytes and bronchial epithelial cells (Lonza) were propagated as adherent cells to plastic in RPMI 1640 medium containing bovine pituitary extract, human epidermal growth factor, insulin, hydrocortisone, gentamicin, and amphotericin at 37°C in a 5% CO_2 incubator. Human primary dermal fibroblasts, HUVEC, HMVEC-L were obtained from Lonza. Macrophages were derived from adherent cells in PBMCs cultured in the presence of 30 ng/ml GM-CSF for 7 d.

RNA isolation and real-time quantitative RT-PCR. Cells were harvested for total RNA isolation using the RNeasy Miniprep kit (QIAGEN), according to the manufacturer's instructions. Total RNA was reverse transcribed using the PrimeScript transcription kit (Takara Bio Inc.). An aliquot of the RT reaction was used as a template for real-time PCR in triplicate using a SYBR Green MasterMix (Takara Bio Inc.) on an Mx3005P thermocycler (Agilent Technologies) with SYBR green I dye as the amplicon detector and ROX as the passive reference. The gene for HPRT was amplified as an endogenous reference. Quantification was determined using both a standard curve and comparative ΔΔCT methods.

ELISA. Conditioned medium from cultured cells was collected after the cells were stimulated and stored at −80°C until use. IL-17A (eBioscience), IL-22 (R&D Systems), IFN-γ, TNF-α, IL-1β, CXCL8 (BD), BD2 (KOMABIOTECH), and BD3 (Alpha Diagnostics) were measured in triplicate by ELISA according to the manufacturers' instructions.

Bactericidal activity against *S. aureus*. *S. aureus* (strain Rosenbach 1884) was obtained from the National Biological Resource Center. Bactericidal activity was evaluated by plating serial dilutions of *S. aureus* mixed with the supernatant from keratinocytes or bronchial epithelial cells, and the CFUs were determined in triplicate on the next day. In some experiments, a neutralizing antibody to BD3 was added to the supernatant.

Chemotaxis. Chemotaxis of neutrophils was determined in triplicate by the Boyden chamber technique. The migration chamber was divided into upper and lower compartments by a membrane with a pore size of 3 μm. The neutrophils were placed into the upper compartment at a concentration of 10^6/ml, and the lower compartment contained the supernatant from the keratinocytes or fibroblasts grown under the conditions indicated. The chambers were incubated at 37° C for 1 h, and the number of neutrophils that migrated to the lower chamber was counted.

Immunoblotting. Cells were lysed on ice for 30 min in lysis buffer containing 1% Triton X-100, 50 mM Tris, pH 8.0, 150 mM NaCl, 2 mM EDTA, 2 μg/ml aprotinin, and 100 μg/ml PMSF. The cell lysates were subjected to SDS-PAGE, followed by electrotransfer to PVDF membranes and immunoblotting with antibodies for IL-1R1, IL-1R2, and IL-1Ra (R&D Systems) and for tubulin (Sigma-Aldrich).

Statistical analysis. Data were compared by a two-tailed Mann-Whitney *U* test or unpaired Student's *t* test. P-values < 0.05 were considered significant.

Online supplemental material. Fig. S1 shows the quantitative RT-PCR analysis for chemokine and BD expression in activated keratinocytes. The importance of IL-17A and IL-22 in stimulating keratinocytes to produce antistaphylococcal factors is demonstrated in Fig. S2. Fig. S3 shows the quantitative RT-PCR analysis for chemokine and BD expression in various types of cells. Production of CXCL8 by keratinocytes and fibroblasts in response to various cytokines is displayed in Figs. S4 and S5. Fig. S6 shows the expression and production of antistaphylococcal factors by keratinocytes in response to various stimuli. Up-regulation of the Th17 cytokine receptors in keratinocytes in response to classical inflammatory cytokines is displayed in Fig. S7. Online supplemental material is available at http://www.jem.org/cgi/content/full/jem.20082767/DC1.

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FBP17 Mediates a Common Molecular Step in the Formation of Podosomes and Phagocytic Cups in Macrophages*[S]

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Macrophages act to protect the body against inflammation and infection by engaging in chemotaxis and phagocytosis. In chemotaxis, macrophages use an actin-based membrane structure, the podosome, to migrate to inflamed tissues. In phagocytosis, macrophages form another type of actin-based membrane structure, the phagocytic cup, to ingest foreign materials such as bacteria. The formation of these membrane structures is severely affected in macrophages from patients with Wiskott-Aldrich syndrome (WAS), an X chromosome-linked immunodeficiency disorder. WAS patients lack WAS protein (WASP), suggesting that WASP is required for the formation of podosomes and phagocytic cups. Here we have demonstrated that formin-binding protein 17 (FBP17) recruits WASP, WASP-interacting protein (WIP), and dynamin-2 to the plasma membrane and that this recruitment is necessary for the formation of podosomes and phagocytic cups. The N-terminal EFC (extended FER-CIP4 homology)/F-BAR (FER-CIP4 homology and Bin-amphiphysin-Rvs) domain of FBP17 was previously shown to have membrane binding and deformation activities. Our results suggest that FBP17 facilitates membrane deformation and actin polymerization to occur simultaneously at the same membrane sites, which mediates a common molecular step in the formation of podosomes and phagocytic cups. These results provide a potential mechanism underlying the recurrent infections in WAS patients.

Podosomes (see Fig. 1*A*) are micron-scale, dynamic, actin-based protrusions observed in motile cells such as macrophages, dendritic cells, osteoclasts, certain transformed fibroblasts, and carcinoma cells (1). Podosomes play an important role in macrophage chemotactic migration, which is critical for recruitment of leukocytes to inflamed tissues. Podosomes are both adhesion structures and the sites of extracellular matrix degradation (2). Adhesion to and degradation of the extracellular matrix are essential processes for the successful migration of macrophages in tissues. Podosomes occur in most macrophages and can be observed by differentiating human primary monocytes into macrophages with macrophage-colony stimulating factor-1 (M-CSF-1)[2] and staining the F-actin using phalloidin (3, 4). Podosomes labeled in this way appear as F-actin-rich dots (see Fig. 1*C*). Podosome formation has recently been directly observed *in vitro* and *in vivo* in leukocyte migration through the endothelium, diapedesis (5).

Phagocytosis of bacterial pathogens is one of the most important primary host defense mechanisms against infections. The phagocytic cup (see Fig. 1*B*) is an actin-based membrane structure formed at the plasma membrane of phagocytes, including macrophages, upon stimulation with foreign materials such as bacteria. The phagocytic cup captures and ingests foreign materials, and its formation is an essential first step in phagocytosis leading to the digestion of foreign materials (6, 7). When macrophages are stimulated by foreign materials, podosomes disappear, and phagocytic cups, which are also rich in F-actin, are formed to ingest the foreign materials (see Fig. 1*D*).

Wiskott-Aldrich syndrome (WAS) is an X chromosome-linked immunodeficiency disorder. Patients with WAS suffer from severe bleeding, eczema, recurrent infection, autoimmune diseases, and an increased risk of lymphoreticular malignancy (8–10). The causative gene underlying WAS encodes Wiskott-Aldrich syndrome protein (WASP) (11). WASP deficiency due to the mutation or deletion causes defects in adhesion, chemotaxis, phagocytosis, and the development of hematopoietic cells in WAS patients (10).

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[2] The abbreviations used are: M-CSF-1, macrophage-colony stimulating factor-1; FBP17, formin-binding protein 17; WAS, Wiskott-Aldrich syndrome; WASP, Wiskott-Aldrich syndrome protein; N-WASP, neuronal WASP; WIP, WASP interacting-protein; EFC domain, extended FER-CIP4 homology domain; F-BAR domain, FER-CIP4 homology and Bin-amphiphysin-Rvs domain; PMA, phorbol 12-myristate 13-acetate; GFP, green fluorescence protein; siRNA, short interfering RNA; FITC, fluorescein isothiocyanate; PDZ-GEF, PDZ-guanine nucleotide exchange factor; HEK293 cells, human embryonic kidney 293 cells; HA, hemagglutinin; SH3, src homology 3 domain; dSH3, SH3 domain deletion; GST, glutathione *S*-transferase; , $PI(4,5)P_2$, phosphatidylinositol 4,5-bisphosphate; siFBP, siRNA for FBP17; siC, scrambled control siRNA.