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Human Skin Mast Cells Express Complement Factors C3 and C5

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We examine whether complement factor C3 or C5 is synthesized by human skin–derived mast cells and whether their synthesis is regulated by cytokines. C3 and C5 mRNAs were assessed by RT-PCR, and proteins by flow cytometry, confocal microscopy, Western blotting, and ELISA. C3 and C5 mRNAs were each expressed, and baseline protein levels/10^6 cultured mast cells were 0.9 and 0.8 ng, respectively, and located in the cytoplasm outside of secretory granules. C3 accumulated in mast cell culture medium over time and by 3 d reached a concentration of 9.4 ± 8.0 ng/ml, whereas C5 levels were not detectable (<0.15 ng/ml). Three-day incubations of mast cells with IL-1α, IL-1β, IL-17, IFN-γ, IL-6, or anti-FcεRI did not affect C3 protein levels in culture medium, whereas incubations with PMA, TNF-α, IL-13, or IL-4 enhanced levels of C3 1.7- to 3.3-fold. In contrast with C3, levels of C5 remained undetectable. Importantly, treatment with TNF-α together with either IL-4 or IL-13 synergistically enhanced C3 (but not C5) production in culture medium by 9.8- or 7.1-fold, respectively. This synergy was blocked by attenuating the TNF-α pathway with neutralizing anti–TNF-α Ab, soluble TNFR, or an inhibitor of NF-κB, or by attenuating the IL-4/13 pathway with Jak family or Erk antagonists. Inhibitors of PI3K, Jnk, and p38 MAPK did not affect this synergy. Thus, human mast cells can produce and secrete C3, whereas β-tryptase can act on C3 to generate C3a and C3b, raising the likelihood that mast cells engage complement to modulate immunity and inflammation in vivo. ***The Journal of Immunology*****, 2013, 191: 1827–1834.**

Mast cells in both humans and mice are recognized as key effector cells of immediate hypersensitivity, and in mice, largely through genetic studies, they regulate an expanded array of innate and acquired immune responses (1–6). The complement system also plays important roles in innate and acquired immune responses (7–11). Some human mast cells, because they express receptors for C3a and C5a, can be activated by these anaphylatoxins to degranulate and produce bioactive lipids, cytokines, and chemokines (12–17). Such mast cells coexpress chymase along with tryptase in their secretory granules and are called the MC_{TC} type of mast cell (18), being essentially the exclusive type of mast cell in skin, but accounting for a minor population of the overall mast cell burden in lung. Most mast cells in lung contain tryptase but not chymase, are unresponsive to complement anaphylatoxins, and are termed the MC_T type of mast cell (19, 20). Stem cell factor (SCF) supports mast cell development, priming, and survival. SCF binds to CD117 (Kit) on the mast cell surface, preventing apoptosis and facilitating their activation (21–24). Human skin mast cells, capable of proliferating when cultured in serum-free medium containing SCF, retain the functional and phenotypic characteristics from when they were freshly dispersed (25). Human mast cell β-tryptase can activate C3 and C5 to generate the corresponding anaphylatoxins (26), delineating a novel putative amplification loop for inflammation initiated by either mast cells or complement (26, 27) and further linking the complement and mast cell pathways to one another in humans. In mice, expression of receptors for C3a or C5a on mast cells is essential not only for acute intradermal responses to C3a or C5a, respectively, but also for an optimal IgE-mediated passive cutaneous anaphylactic response to allergen (28).

Although most of the C3 and C5 in the circulation are produced by the liver, their extrahepatic production by various cell types, including macrophages, dendritic cells, fibroblasts, epithelial cells, endothelial cells, keratinocytes, smooth muscle cells, and neuronal cells, either spontaneously or in response to cytokine stimulation, has been well documented (29–41). Locally synthesized C3 and C5, presumably through their bioactive metabolites, importantly regulate other aspects of inflammation and host defense (42, 43), for example, clearance of immune complexes by macrophages (44). As studied with APCs in murine systems, C3 and C5 affect expression of costimulatory molecules in an autocrine manner and T cell development in a paracrine manner (41, 45–48), thereby modulating murine immunity along with models of inflammatory bowel disease (49), multiple sclerosis (50), and graft-versus-host disease (10). These studies show the importance of locally produced and activated complement proteins.

Synthesis of complement factors C3 and C5 by human mast cells has not been previously reported. However, mice are more likely to succumb to sepsis caused by cecal ligation and puncture if they are deficient either in C3 (51) or in mast cells (1). Moreover, mast cells from C3-deficient mice exhibit reduced degranulation (51), and mice whose mast cells are deficient in C3aR or C5aR show reduced IgE-dependent degranulation and inflammation in skin (52). To further examine the complement/mast cell axis in humans, we studied the expression of factors C3 and C5 by human primary skin mast cells, showing that both are expressed, only C3 is secreted, and production of C3 is synergistically upregulated by TNF-α together with either IL-4 or IL-13.

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Abbreviations used in this article: MC_T, mast cell containing tryptase but not chymase; MC_{TC}, mast cell coexpressing chymase along with tryptase; PFTα, cyclic-pifithrin-α; qRT-PCR, quantitative real-time PCR; SCF, stem cell factor; VCU, Virginia Commonwealth University.

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Materials and Methods

Reagents

Mouse IgG anti-human C3/C3b mAb and biotin-rabbit IgG anti-human C3 (Abcam, Cambridge, MA); mouse IgG anti-human C5/C5b mAb (Cell Sciences, Canton, MA); sheep IgG anti-human C3c Ab, sheep IgG anti-human C5 Ab, and rabbit anti-C3 Ab (GeneTex, Irvine, CA); goat IgG anti-human C5 (Complement Technology, Tyler, TX); goat IgG anti-human TNF-α Ab, goat IgG anti-human C3, recombinant human soluble TNFR, purified human C3, C5, C3a, recombinant human C5a, Jak inhibitor 1, cyclic-pifithrin-α (PFTα), tyrphostin AG9, U0126, SB203580 (EMD Millipore, Billerica, MA); human C5a (Complement Technology, Tyler, TX); soybean trypsin inhibitor, PMA, wortmannin (Sigma-Aldrich, St. Louis, MO); human IL-1α, IL-1β, IL-4, IL-6, IL-13, IL-17, TNF-α, and IFN-γ (R&D Systems, Minneapolis, MN); and SP600125, CAY10470 (Cayman Chemical Company, Ann Arbor, MI) were obtained as indicated. Mouse anti-human tryptase mAb, B12, was prepared in-house (53); mouse IgG anti-FcεRIα mAb (22E7) was generously provided by J. P. Kochan (Hoffman-LaRoche, Nutley, NJ) (54). U-937 cells (human monocytic cell line), A549 cells (human lung carcinoma epithelial cell line), and HMC-1 cells (human mast cell line) were cultured in RPMI-1640, DMEM, and Iscove's medium, respectively, supplemented with 10% FBS, 2 mM L-glutamine, 100 U/ml penicillin, and 100 mg/ml streptomycin at 37°C, 5% CO_2 in a humidified incubator.

Isolation and culture of human MC_{TC} cells and MC_T cells

Human skin mast cells and lung mast cells were isolated as described previously (25, 55). Fresh skin and lung were obtained from the Pathology Department of Virginia Commonwealth University (VCU) or Cooperative Human Tissue Network of the National Cancer Institute or National Disease Research Interchange as reviewed and approved by the VCU Institutional Review Board, and used as a source of mast cells. Skin was cut in fragments and incubated in a solution of HBSS containing type 2 collagenase (1.5 mg/ml; Worthington Biochemicals, Lakewood, NJ), hyaluronidase (0.7 mg/ml; Sigma-Aldrich), type 1 DNase (0.3 mg/ml; Sigma-Aldrich), 1% FCS, and 1 mM $CaCl_2$ for 2 h at 37°C. The dispersed cells were separated from residual tissues by filtration through #80 mesh sieve and suspended in HBSS containing 10 mM HEPES and 1% FCS. Cells were layered over 75% Percoll (Sigma-Aldrich) and centrifuged at 450 × *g* at room temperature for 20 min. Nucleated cells were collected from the buffer/Percoll interface, and skin mast cells (MC_{TC} cells) were resuspended in serum-free X-VIVO-15 Media (Lonza, Allendale, NJ) with 100 ng/ml recombinant human SCF (a gift from Swedish Orphan Biovitrum, Stockholm, Sweden), and cultured up to 2–3 mo. Major contaminated cells are adherent cells including fibroblasts and macrophages. These adherent cells are removed by transferring nonadherent mast cells to another culture plate. To purify freshly dispersed skin mast cells, we cultured such cells overnight to recover from the dispersion process; then they were positively selected with mouse IgG anti-Kit (CD117)–conjugated magnetic beads using an MACS system (Miltenyi Biotec, Auburn, CA).

Lung mast cells (primarily MC_T cells) were obtained as described previously (55) by protease digestion and Percoll density-dependent sedimentation. MC_T cells were purified by depleting MC_{TC} cells with mouse anti-CD88 Ab and rat anti-mouse IgG-conjugated magnetic beads using an MACS system and then positively selecting the remaining mast cells using mouse IgG anti-Kit–conjugated magnetic beads. MC_T cells were cultured in serum-free X-VIVO-15 Media and 100 ng/ml SCF. The purities of skin and lung mast cells were routinely analyzed by toluidine blue staining and confirmed by flow cytometry using anti-FcεRI or anti-CD117 Abs.

Flow cytometry and confocal microscopy

Intracellular labeling of skin mast cells by mouse anti-C3/C3b mAb or by mouse anti-C5/C5b mAb was performed using Cytofix/Cytoperm Fixation/permeabilization Kit (BD Biosciences, San Jose, CA) and followed by incubation with PE-labeled goat anti-mouse IgG. Stained cells were assessed by flow cytometry (FACSCanto II using CellQuestPro software; BD Biosciences). For confocal microscopy analysis, permeabilized human skin mast cells were labeled with C5- or C3-specific primary mAbs followed by FITC-goat anti-mouse IgG. Stained cells were attached to poly-L-lysine-treated glass microscope slides and mounted (Vectashield Hard set; Vector Laboratories, Burlingame, CA). Confocal microscopy measurement was performed at VCU Microscopy Facility (Dr. Scott Henderson, Director) in the Department of Anatomy and Neurobiology using a Leica TCS-SP2 AOBS confocal laser scanning microscope (Leica Microsystems, Buffalo Grove, IL).

Western blotting and immunoprecipitation

Skin mast cells were extracted using 10 mM Tris-HCl, pH 7.4, buffer containing 150 mM NaCl, 1 mM EDTA, 0.5% sodium deoxycholate, 1% Nonidet P-40, 0.1% SDS, and Protease Inhibitors Set (Roche Applied Science, Indianapolis, IN) for 1 h at 4°C and centrifuged. Supernatants were mixed with SDS-sample buffer containing 2% 2-ME, boiled for 5 min, and subjected to electrophoresis on an 8–16% polyacrylamide gradient gel containing SDS. Proteins were transferred onto a nitrocellulose membrane using a Novex system (Life Technologies, Long Island, NY) for 1 h at 50 V. After applying blocking buffer (LI-COR Biosciences, Lincoln, NE) for 1 h at room temperature, membranes were labeled with each primary Ab for 1 h at room temperature, followed by a 1-h incubation with an IRDye 800CW-conjugated secondary Ab (LI-COR Biosciences). Labeling was analyzed using an Odyssey infrared imaging system (LI-COR Biosciences). For immunoprecipitation of C3, rabbit anti-C3 Ab was incubated with Protein A/G PLUS-Agarose (Santa Cruz Biotechnology, Santa Cruz, CA) for 1 h, washed, and added to 30 ml skin mast cell culture medium with Protease Inhibitors Set for 1 h at room temperature. Immunoprecipitates were mixed with SDS-sample buffer containing 2% 2-ME, boiled for 5 min, and subjected to electrophoresis on a 10% polyacrylamide gel containing SDS. Proteins were transferred onto a nitrocellulose membrane. After blocking with PBS, pH 7.4, containing 5% BSA and 0.05% Tween 20 for 1 h, the membrane was probed with goat IgG anti-human C3 for 1 h at room temperature followed by incubations with alkaline phosphatase–conjugated donkey IgG anti-goat IgG (Jackson Immunoresearch Laboratories, PN) and developed with a 5-bromo-4-chloro-3-indolyl phosphate/NBT alkaline phosphatase liquid substrate solution (Sigma-Aldrich).

Quantitative real-time PCR

Quantitative real-time PCRs (qRT-PCRs) were performed with the CFX96 Real-Time system using a C1000 Touch Thermal cycler (Bio-Rad, Hercules, CA) and QPCR SYBR Green Low ROX mixes (Thermo Fisher Scientific, Waltham, MA). Total RNA was isolated from mast cells by RNAeasy Mini kit (Qiagen, Valencia, CA), and 1 μg RNA was treated by RNase-free DNase (Promega, Madison, WI) at 37°C for 1 h. After denaturation at 65°C for 10 min, cDNA was synthesized using the Ready-To-Go You-Prime First-Strand Beads for cDNA synthesis (GE Health Care Biosciences, Pittsburgh, PA) with oligo(dT) primer (Life Technologies) for 1 h at 37°C. Reaction samples include 10 μl SYBR green/Low ROX PCR Master mix, 0.5 μl of primer set, 2 μl cDNA, and 7.5 μl water in the wells of 98-well microplates. The cycling condition was 50°C for 2 min, 95°C for 10 min, 40 cycles of 95°C for 15 s, 60°C for 30 s, and 68°C for 30 s, and a final extension at 68°C for 10 min. Each of the standard PCR products was diluted 10^{-4}, 10^{-5}, 10^{-6}, 10^{-7}, and 10^{-8}. The amount of cDNA was determined from the standard curve made by each value of threshold cycle and normalized by dividing the β-actin threshold cycle value. Primer pairs for C3, C5, and β-actin (synthesized at VCU DNA core facility) were as follows: C3 (186 bp), sense 5′-TCACCGTCAACCACAAAGCTGCTACC-3′ and antisense 5′-TTTCATAGTAGGC TCGGATCTTCCA-3′; C5 (188 bp), sense 5′-GTTGAAGCCCGAGAGAACAG-3′ and antisense 5′-AGGGAAAG-AGCATACGCAAGA-3′; β-actin (127 bp), sense 5′-AGGCACCAGGG-CGTGAT-3′ and antisense 5′-TGCTCCCAGTTGGTGACGAT-3′.

C3 and C5 ELISAs

C3 and C5 protein levels, respectively, were measured by ELISA using 1 μg/ml sheep anti-C3c and sheep anti-C5 Abs for capture and 1 μg/ml biotin-rabbit IgG anti-C3 and biotin-goat IgG anti-C5 (goat IgG anti-human C5 IgG was purified by protein G-Sepharose chromatography from antiserum prepared by Comp Tech and was then conjugated with biotin in-house) Abs for detection. After incubation with avidin-peroxidase (1:1000 dilution; BD Biosciences), 2.2-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt solution (0.3 mg/ml; Sigma-Aldrich) was added and the absorbance at 405 nm was measured using a SpectraMax 384 Plus plate reader (Molecular Devices, Sunnyvale, CA). The lower detection limit for each protein was 0.15 ng/ml.

Statistics

Statistical analyses were performed using SigmaStat (Systat Software, Chicago, IL). For parametric data, Student *t* test for two-group and ANOVA for more than two-group comparisons were used, whereas for nonparametric data, a Kruskal–Wallis ANOVA on ranks was used to compare more than two groups. Differences were considered statistically significant when p values were < 0.05.

Results

C3 and C5 proteins are produced by skin mast cells

To detect C3 and C5 proteins in human skin mast cells, we fixed, permeabilized, labeled with anti-C3/C3b, anti-C5/C5b, or

isotype-matched negative control Ab, and assessed these cells by flow cytometry. As depicted in Fig. 1A, both anti-C3 and anti-C5 Ab showed a positive signal compared with isotype control. To confirm the specificity of the shift, we showed purified human C3 or C5 to compete with the corresponding Ab (Fig. 1B), consistent with C3 and C5 being present within these mast cells. Confocal analysis showed a diffuse cytoplasmic labeling pattern with both anti-C3 and anti-C5 Abs (Fig. 1C), unlike the granular pattern seen with Abs against tryptase, a well-recognized component of the mast cell secretory granule (56, 57). Mast cells stimulated with anti-FcεRI Ab for 15 min secreted β-hexosaminidase, another secretory granule marker in mast cells (58, 59), but negligible amounts of C3 or C5 (Fig. 1D), confirming the subcellular localization of cytoplasmic C3 and C5 being outside of the secretory granules. Importantly, having cultured these mast cells for 6–12 wk in serum-free medium containing no detectable C3 and C5 (<0.15 ng/ml), exogenous complement factors cannot account for the C3 and C5 detected, supporting their production by these mast cells.

To further examine the C3 and C5 protein produced by skin mast cells, we performed Western blotting with protein extracts, using sheep anti-human C3 Ab and biotin-goat anti-human C5 Ab (Fig. 1E). Both C3α- and β-chains and C5α- and β-chains were evident in skin mast cell extracts, with molecular masses comparable with those detected in extracts of A549 and HMC-1 cells and with those in purified preparations of C3 and C5. C3 and C5 proteins secreted into the mast cell culture medium also were examined. Immunoprecipitation from culture medium was performed using rabbit IgG anti-human C3 or anti-human C5 bound to protein A/G-agarose to analyze these proteins. As shown in Fig. 1F, eluates from the anti-C3:protein A/G-agarose step revealed C3α- and C3β-chains. Interestingly, the C3α-chain was partially cleaved. In contrast, C5 protein was not recovered from mast cell culture medium by immunoprecipitation. Levels of C3 and C5 produced by skin mast cells were more precisely quantified by ELISA. Respective C3 and C5 levels in extracts of skin mast cells were 0.9 ± 0.1 and 0.8 ± 0.3 ng per 10^6 cells, whereas those in extracts of A549 were 2.2 ± 0.3 and 25.2 ± 4.8 ng per 10^6 cells. The time course of C3 accumulation in the mast cell culture medium, measured by ELISA, is shown in Fig. 1G. The C3 level increased from <0.15 ng/ml at time 0 to 8.6 ± 1.4 ng/ml on day 3. C3 levels of U937 cell and A549 cell culture medium increased to 10–50 and 10–20 ng/ml on day 3, respectively (data not shown). Consistent with the immunoprecipitation/Western blot finding for secreted C5 noted earlier, the C5 level in mast cell culture medium after 3 d remained at <0.15 ng/ml. In contrast, C5 levels in A549 cell culture medium were 2.5 ng/ml on day 1 and 18.2 ng/ml on day 5 (data not shown).

To address the possible effect of culture condition on C3 and C5 expression, we compared C3 expression by skin mast cells in culture for 2–3 mo with those that were freshly dispersed and isolated. As shown in Table I, C3 and C5 proteins and mRNAs were detected in mast cells from both groups, and comparable amounts of C3 were secreted over a 3-d interval. Thus, expression of C3 and C5 by human skin–derived mast cells is not an artifact of long-term culture.

C3 and C5 mRNA expression

Conventional RT-PCR was performed to establish the presence C3 and C5 mRNA. As shown in Fig. 2A, the predicted sizes of the C5 (188 bp) and of the C3 (186 bp) RT-PCR products were detected in skin mast cell–derived cDNA, as well as in U937 cDNA and HMC-1 cDNA. Expression of C3 and C5 mRNA by lung and skin mast cells were compared by qRT-PCR (Fig. 2B). Median levels of C3 mRNA levels in skin and lung mast cells were not signifi-

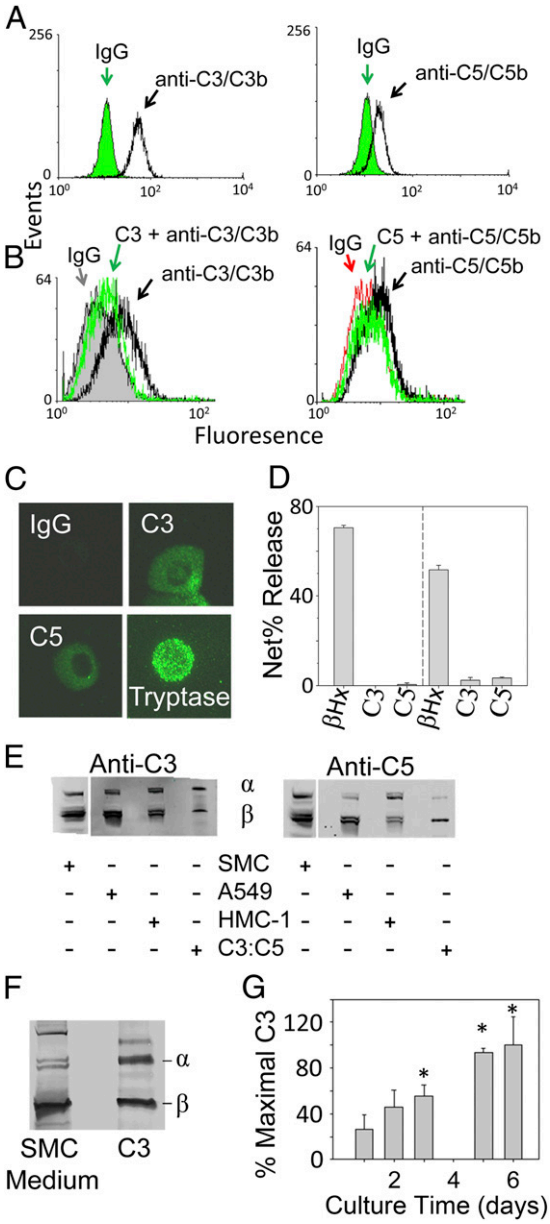

FIGURE 1. C3 and C5 protein expression in human skin mast cells. (**A**) Detection of C3 and C5 in fixed and permeabilized human skin mast cells labeled with anti-C3/C3b or anti-C5/C5b Ab by flow cytometry. (**B**) Purified C3 and C5 compete with labeling with anti-C3/C3b and anti-C5/C5b Abs, respectively, as in (A). (**C**) Confocal microscopy of human fixed and permeabilized mast cells were labeled first with nonimmune mouse IgG, IgG anti-C3/C3b (C3), IgG anti-C5/C5b (C5), anti-tryptase (tryptase) Ab, and then with FITC-conjugated goat anti-mouse IgG Ab (original magnification ×500). (**D**) Release of β-hexosaminidase, C3, and C5 by mast cells stimulated for 15 min with 22E7 (100 ng/ml) in three replicates from two different skin preparations. (**E**) Western blot analysis of C3 and C5 proteins expressed by human skin mast cells. Cell extracts (2×10^6 skin mast cells and 1×10^6 A569 and HMC-1 cell equivalents/lane); purified human C3 and C5 (10 ng/lane). (**F**) C3, spontaneously secreted into the culture medium, was immunoprecipitated with rabbit IgG anti-C3 and detected by Western blotting with goat IgG anti-C3 Ab (*lane 1*). The α (120 kDa) and β (75 kDa) subunits of commercial C3 are shown in *lane 2*. (**G**) Unstimulated skin mast cells in culture produce C3 in a time-dependent manner. Medium from cultured skin mast cells (10^6 cells/ml) containing 100 μg/ml soybean trypsin inhibitor was collected at the times indicated and assessed for C3 content by ELISA. Medium alone contained <0.15 ng/ml C3. Data shown are the average of four independent experiments, each one normalized to the maximal amount of C3 for that experiment. *$p < 0.05$, comparing values on days 1–6 of culture with medium at time 0 by ANOVA.

Table I. Comparisons of cultured and freshly dispersed skin mast cells

Skin Mast Cells	Cultured	Freshly Dispersed
C3 flow cytometry (net MFI)	115 ± 25 (n = 5)	44 ± 18 (n = 5)
Secreted C3 (ELISA, ng/ml)	2.5 ± 0.5 (n = 13)	2.5 ± 0.6 (n = 9)
C3 mRNA (qRT-PCR, fold increase)	1.0 ± 0.3 (n = 3)	2.5 ± 0.3* (n = 4)
C5 flow cytometry (net MFI)	61 ± 7.4 (n = 3)	31 ± 10.1 (n = 3)
C5 mRNA (qRT-PCR, fold increase)	1.0 ± 0.2 (n = 3)	1.0 ± 0.1 (n = 3)

*$p < 0.05$.

cantly different from one another and were comparable with those in U937 cells, as was also the case for C5 mRNA levels.

TNF-α together with IL-4 or IL-13 synergistically enhance C3 production

The effect of cytokines reported to stimulate C3 synthesis in cells other than mast cells, including IL-1α, IL-1β, IL-4, IL-6, IL-13, IL-17, TNF-α, and IFN-γ, were examined with human skin mast cells. Although exposure to PMA for 3 d significantly increased levels of C3 in the culture medium, neither IL-1α, IL-1β, IFN-γ, nor aggregation of FcεRI (22E7 mAb) had such an effect (Fig. 3A). Levels of C3 mRNA also were unaffected by stimulation with 22E7 for 4 h (data not shown). IL-6, as well as the combination of IL-1β with IL-6, also failed to affect C3 levels (Fig. 3A). TNF-α, IL-4, and IL-13 stimulation each enhanced C3 levels 2- to 3-fold above those associated with unstimulated cells (Fig. 3B, 3C). Importantly, the combination of TNF-α with IL-4 and of TNF-α with IL-13 synergistically enhanced C3 levels 9.8 ± 6.6 and 7.1 ± 5.1-fold, respectively. IL-17 had no effect on C3 levels, either by itself or in combination with TNF-α. C5 protein again was not detected in culture medium after exposure to these cytokine combinations. To confirm that the synergistic effect of TNF-α on C3 was, in fact, due to the TNF-α in these commercial preparations, we blocked TNF-α protein by either anti–TNF-α Ab or recombinant soluble TNF-αR. In each case, the synergetic effect of TNF-α and IL-4 was completely blocked by these treatments (Fig. 3D).

To examine the signaling pathways involved in cytokine-stimulated C3 production, we examined the effects of inhibitors of PI3K and MAPK (Fig. 4A). The synergistic effect of TNF-α together with either IL-13 or IL-4 was significantly, but not completely, inhibited by an Erk (U0126) inhibitor, but not by a PI3K (wortmannin), a JNK (SP600125), or a p38 MAPK (SB203580) inhibitor. JNK reportedly is involved in TNF-α signaling and Erk in IL-4/IL-13 signaling.

Because the NF-κB pathway plays a major role in TNF-α signaling, the effect of an NF-κB inhibitor (CAY10470) was examined. As shown in Fig. 4B, the synergistic effect of TNF-α

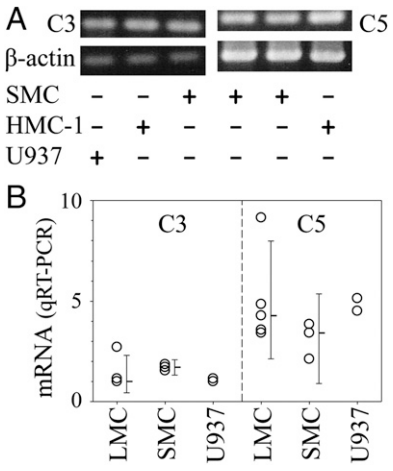

FIGURE 2. C3 and C5 mRNA expression in human skin mast cells. (**A**) RT-PCR analysis of human skin mast cells. The sizes of the products are 186 (C3), 188 (C5), and 127 bp (β-actin). Extracts of human skin mast cells, HMC-1 cells, and U937 cells were obtained and subjected to RT-PCR using the same primers and cycling parameters as for qRT-PCR, except termination occurred after 35 cycles and Taq DNA polymerase was used. (**B**) Relative amount of C3 and C5 mRNA levels of lung mast cells (LMC) and human skin mast cells by qRT-PCR. Levels of C3 and C5 mRNAs were measured by qRT-PCR and normalized to β-actin. U937 cells served as a positive control. Bars indicate the median values of C3 and C5 levels; error bars indicate the 95% confidence intervals. Median values of C3 or C5 were compared between skin and lung mast cells using a Mann–Whitney rank sum test.

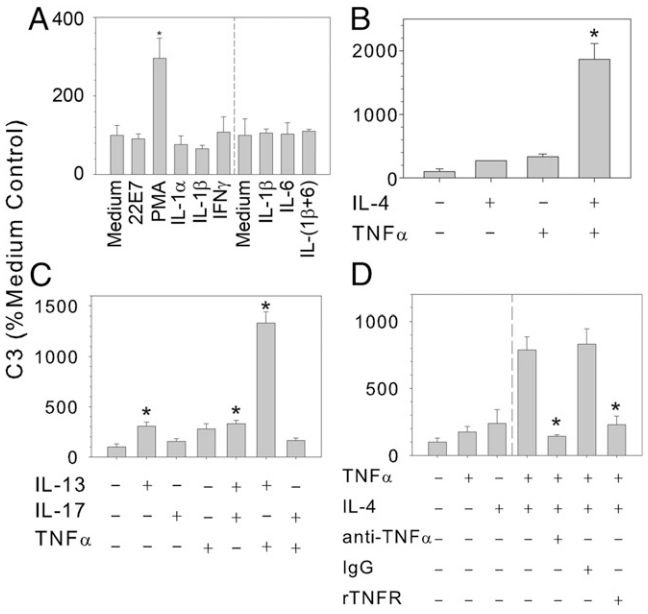

FIGURE 3. Effect of cytokines on C3 synthesis by human skin mast cells. Th1, Th2, and Th17 cytokines and PMA can increase C3 production by skin mast cells. Skin mast cells (10^6 cells/ml) were treated with each stimulant for 3 d with 100 μg/ml soybean trypsin inhibitor, and C3 concentrations in culture medium were measured by ELISA. (**A**) Medium alone, 22E7 (10 ng/ml), PMA (10 ng/ml), IL-1α (10 ng/ml), IL-1β (10 ng/ml), IFN-γ (100 ng/ml), and IL-6 (10 ng/ml) were used as indicated, with different mast cell preparations being used in the experiments depicted to the left and right of the dashed line. (**B**) IL-4 (5 ng/ml) and TNF-α (10 ng/ml) were used as indicated. (**C**) IL-13 (100 ng/ml), IL-17 (100 ng/ml), and TNF-α (10 ng/ml) were used as indicated. (**D**) Anti–TNF-α Ab (5 μg/ml; isotype-matched control IgG at 5 μg/ml) and recombinant soluble TNF-αR (2.5 μg/ml) each block the synergistic effect of TNF-α (10 ng/ml) on IL-4 (5 ng/ml)–stimulated production of C3 by skin mast cells. Data shown are the average of at least three independent experiments. *$p < 0.05$ in (A)–(C) for comparing various triggers with medium by ANOVA and in (D) for comparing the effects of anti–TNF-α IgG, control IgG, or rTNF-αR on C3 production by IL-4 and TNF-α (data to right of dashed line).

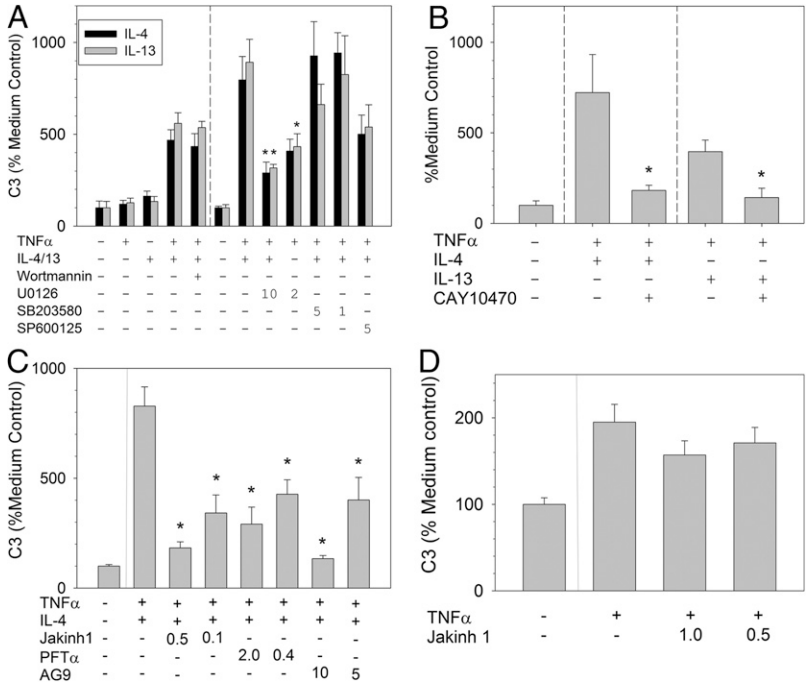

FIGURE 4. Effect of signal inhibitors on synergistic enhancement of C3 production by human skin mast cells. (**A**) The synergistic enhancement of C3 production by human skin mast cells exposed to TNF-α together with IL-13 or IL-4 is partially inhibited by Erk inhibitor (U0126), but not by a PI3K (Wortmannin), Jnk (SP600125), or p38 MAPK (SB203580) inhibitor. Human skin mast cells were pretreated with each inhibitor for 1 h and stimulated with each cytokine for 3 d. The levels of C3 in culture supernatant were measured by ELISA. TNF-α (10 ng/ml), IL-13 (100 ng/ml), IL-4 (5 ng/ml), Wortmannin (100 nM), U0126 (10, 2 μM), SB203580 (5, 1 μM), and SP600125 (5 μM) were used at the concentrations indicated. Data to the left and to the right of the dashed vertical line represent separate experimental groups. $*p < 0.05$, comparing by ANOVA the effect of each inhibitor on C3 production by TNF-α plus either IL-4 or IL-13. (**B**) Effect of NF-κB inhibitor on C3 production by TNF-α together with either IL-13 or IL-4 from human skin mast cells. TNF-α (10 ng/ml), IL-13 (100 ng/ml), IL-4 (5 ng/ml), and CAY 10470 (1 μM) were used at the concentrations indicated. $*p < 0.05$, using a Student *t* test to compare ± CAY10470. (**C**) Inhibitors of the Jak/STAT pathway inhibit the synergistic effect of IL-4 and TNF-α on C3 production by human skin mast cells. TNF-α (10 ng/ml), IL-4 (5 ng/ml), Jak inhibitor 1 (0.5 and 0.1 μM), PFTα (2 and 0.4 μM), and tyrphostin AG9 (10 and 5 μM) were used as indicated. Data shown are the average of at least three independent experiments. $*p < 0.05$ comparing effects of inhibitors on C3 production by TNF-α plus IL-4 by ANOVA. (**D**) Effect of Jak inhibitor 1 (1 and 0.5 μM) on TNF-α (10 ng/ml)–stimulated C3 production from human skin mast cells. Data shown are the average of at least three independent experiments. The effects of inhibitors on C3 production triggered by TNF-α were examined by ANOVA.

together with either IL-4 or IL-13 was completely inhibited. Because only the IL-4Rα/IL-13Rα1 receptor is stimulated by both IL-4 and IL-13, and this receptor signals through Tyk2 and Jak1, the effect of Jak inhibitor-1 was examined. As shown in Fig. 4C, the synergistic effect of IL-4 together with TNF-α was almost completely inhibited by Jak inhibitor-1. Because Jak inhibitor-1 is promiscuous among all Jak family members, including Tyk2, the effect of a Tyk2-specific inhibitor, tyrphostin AG9, was examined. Tyrphostin AG9 also inhibited the synergetic effect of IL-4, indicating the likely involvement of IL-13Rα1–associated Tyk2 signaling. Inhibition of STAT6, a downstream signaling molecule of the IL-4 response, by PFTα also inhibited the synergy of TNF-α and IL-4. To examine whether the Jak/STAT pathway is also involved in TNF-α signaling in human skin mast cells, we examined the effect of Jak inhibitor 1 on TNF-α–mediated IL-6 synthesis; IL-6 response was significantly inhibited by 26 ± 7 and 51 ± 8% at 0.5 and 1 μM Jak inhibitor 1, respectively, indicating that TNF-α signals, in part, throw a Jak/STAT pathway (data not shown). Fig. 4D shows that the small increment in C3 synthesis caused by TNF-α stimulation was also inhibited, by only 25 and 40% at 0.5 and 1 μM Jak inhibitor 1, respectively, and these levels of inhibition were not statistically significant.

Discussion

Complement proteins and mast cells cross-interact with one another and bridge both innate and adaptive immune responses. Human mast cells of the MC_{TC} type, essentially the exclusive type in skin, are distinguished from the MC_T type, the dominant type in lung parenchyma and small-bowel mucosa, by expressing both tryptase and chymase instead of tryptase alone, and by being responsive to the anaphylatoxins C5a and C3a, secreting both preformed and newly generated mediators when exposed to these anaphylatoxins, a functional phenotype that is maintained during the serum-free culture conditions used in this study. Moreover, mast cell tryptase can generate C3a and C5a from their respective C3 and C5 precursors. This study extends this relationship by showing for the first time, to our knowledge, that both C3 and C5 are produced by primary skin-derived human mast cells.

C3 and C5 were constitutively expressed by human skin mast cells, as shown by detecting C3 and C5 mRNAs and proteins. Skin mast cells (MC_{TC} type) and lung mast cells (predominantly MC_T type) show similar levels of C3 and C5 mRNAs. Flow cytometry and confocal microscopy, each using fixed and permeabilized cells, as well as Western blotting, using cell extracts, detected C3 and C5 proteins. The 120-kDa α and 75-kDa β subunits of C3 and C5 migrated with the same electrophoretic mobilities as those derived from certain cell lines and as the commercial purified proteins, confirming their identities. The amounts of C3 and C5 measured in mast cell extracts by ELISAs were 0.9 and 0.8 ng/10^6 cells, respectively. Whereas confocal microscopy localized tryptase to cytoplasmic granules, C3 and C5 both exhibited a diffuse cytoplasmic pattern, indicating they were not localized to secretory granules. In

support of their localization outside of secretory granules, degranulation of skin mast cells with anti-FcεRI Ab did not stimulate secretion of C3 or C5. Instead, C3 was constitutively secreted, its concentration in culture medium steadily increasing from <0.15 ng/10^6 cell equivalents in the initial medium to 8.6 ng/10^6 cell equivalents by day 3 and ~17 ng/10^6 cell-equivalents by day 6, indicating that more C3 was secreted within a week than remained inside these cells. In contrast, C5 was not detected in the culture medium at any time point through 6 d, remaining at <0.15 ng/10^6 cells. Whether C5 might have an intracellular role or simply has a yet to be identified secretion signal remains to be determined. Secreted C3, when Western blotted, exhibited a doublet near the α subunit, likely corresponding to α and α′, indicating C3 cleavage during or after secretion. Examples of proteases that can generate C3a from C3 in serum-free medium include mast cell tryptase (26) and granzyme B (60), resulting in C3b (α′β). Mast cell proteases other than tryptase, including membrane-bound metalloproteases, also might have a role in activating C3 and C5. Proteases in mouse Kupffer cells (44), rat alveolar macrophages, and human neutrophils (61), and in a human kidney epithelial cell line (62) process C5 or C3 in a similar manner. Although skin mast cells are typically cultured in serum-free medium with SCF for 2–3 mo before being used experimentally, these culture conditions did not account for the production of C3 and C5, because comparable mRNA and protein levels were found in freshly dispersed and purified skin mast cells. Moreover, levels of C3 secretion from freshly isolated skin mast cells were similar to those that had been in culture for 2–3 mo.

Several inflammatory cytokines, each by themself, are known to enhance C3 or C5 protein levels, depending on the cell type and species, including IL-1α, IL-1β, IL-4, IL-6, IL-17, TNF-α, and IFN-γ (31–34, 36–39, 63–65). Whether cytokines might influence the amounts of C3 or C5 secreted by human skin mast cells was experimentally addressed. Unlike reported for fibroblasts, neither C3 (Fig. 3A,C) nor C5 (data not shown) levels in skin mast cells were affected by IL-1α, IL-1β, IL-17, or IFN-γ. C3 is a type I acute-phase glycoprotein, induced by IL-6 and IL-1 in combination better than by either alone (66–68), for example, in human skin fibroblasts (69, 70) or rat hepatoma cells (64). Indeed, both IL-1– and IL-6–responsive elements have been identified in human and mouse C3 promoter genes (71, 72). However, neither IL-6 nor IL-1β, nor the combination of these cytokines, induced significant C3 production by skin mast cells, indicating response differences among distinct cell types and/or species.

IL-4, IL-13, or TNF-α modestly enhanced secreted levels of C3 (but not those of C5). However, when combinations of IL-4 or IL-13 with TNF-α were assessed, a remarkable synergism became evident, dramatically increasing secretion of C3 by mast cells (but again not affecting that of C5). The combination of TNF-α with either IL-13 or IL-4 also enhanced C3 production by human fibroblasts (38), although the mechanism was not addressed. The synergy from combining IL-13 or IL-4 with TNF-α could be blocked with either neutralizing anti–TNF-α Ab, with recombinant TNFR, or with an inhibitor of NF-κB, indicating dependence on TNF-α activity and its downstream activation of NF-κB. Because activated mast cells can produce TNF-α and IL-13, such cells may enhance local C3 production by autocrine and paracrine pathways, with paracrine pathways potentially affecting both mast cells and nonmast cells.

IL-13 acts primarily on the IL-4Rα/IL-13Rα1 heterodimer, whereas IL-4 can act on this receptor, as well as the IL-4Rα/γc heterodimer. Human mast cells express both IL-4Rα/γ_c and IL-4Rα/IL-13Rα1 (73), with IL-4 known to enhance mediator release by different types of human mast cells (74–76). Triggering either of these receptors activates Jak1/STAT6 and the PI3K and Ras/MAPK pathways (77–80). The human C3 promoter has STAT6 binding sites (81, 82), consistent with the observed upregulation of C3 expression. The IL-13Rα1 subunit, but not the IL-4Rα and γ_c subunits, reportedly signal through Tyk2 (79). The IL-4/13–TNF-α synergies were attenuated with the Tyk2-specific inhibitor Tyrphostin AG9 almost as well as with the Jak inhibitor-1 (which inhibits all four members of the Jak family, including Tyk2) (78–80), indicating that the IL-4Rα/IL-13Rα1 heterodimer accounts for most, if not all, of the synergy added by IL-4 or IL-13 to TNF-α. However, because TNF-α signaling also involves the Jak/STAT pathway (83, 84), TNF-α may act, in part, through this pathway. It is also possible TNF-α–induced Jak/STAT activation enhances IL-4R–mediated Jak/STAT signaling. The lack of an effect by Wortmannin, SP600125, or SB203580 argues that neither PI3K, Jnk, nor p38 MAPK, respectively, is involved in the synergistic production of C3, whereas partial inhibition by U0126 indicates Erk involvement. Because TNF-α signals in part through JNK (85, 86), whereas IL-4 and IL-13 signal in part through Erk (80, 87), it is not surprising that Erk clearly contributes to this synergism, whereas the lack of effect of a Jnk inhibitor suggests TNF-α involvement does not involve Jnk.

Mast cells certainly encounter IL-4/13 and TNF-α at sites of inflammation, such as the asthmatic airways and atopic dermatitis skin, themselves being an important source of IL-13. Increased production of C3 by mast cells and conversion of C3 to C3a and C3b at these sites, perhaps by mast cell tryptase, might further modulate inflammation and immunity. Local production of C3 by dendritic cells in mice, for example, promotes Th1 development (41, 47). C3a can amplify pathologic process by increasing vasopermeability, contracting bronchial smooth muscle, stimulating dendritic cells to modulate T cell activation, or activating mast cells and basophils, of likely importance in allergic disorders such as asthma and in autoimmune urticaria (88–98). Furthermore, C3adesArg, a metabolite of C3a, serves as an adipokine associated with obesity, type II diabetes, and coronary artery disease (99). Indeed, mast cell numbers are increased in the white adipose tissues of obese subjects (100) and the bronchial smooth muscle of asthmatics (101), providing them with the opportunity to influence disease activity by both producing C3 and, through tryptase or other proteases, converting C3 to C3a and C3adesArg. When C3 is cleaved to yield C3a, C3b presumably is also generated, exposing its reactive thioester domain, thereby allowing covalent binding to nearby proteins or carbohydrates, opsonizing those targets, enhancing their immunogenicity and their susceptibility to phagocytosis or endocytosis (11). Thus, this newfound ability of human mast cells to produce C3 has implications for their involvement in diseases ranging from asthma to obesity.

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Disclosures

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