

Comparative cytokine profile of human skin mast cells from two compartments—strong resemblance with monocytes at baseline but induction of IL-5 by IL-4 priming

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Abstract: Although known as heterogeneous, mast cells (MC) are believed to induce allergic inflammation, partially by secretion of T helper cell type 2 (Th2) cytokines. We show here that MC purified from two human skin compartments produce cytokines that are primarily associated with inflammation and innate immunity [interleukin (IL)-1 β , IL-6, IL-8, tumor necrosis factor α (TNF- α)]. Although these are detectable even without stimulation, immunoglobulin (Ig)E receptor cross-linking is able to enhance only TNF- α production, but phorbol 12-myristate 13-acetate additionally promotes IL-1 β and IL-8. With the exception of TNF- α , the presence of serum has a positive impact on cytokine production. Although IL-13 transcripts (but not those for IL-4 and -5) are produced by skin MC, all Th2 cytokines remain undetectable in the supernatants or lysates of MC from foreskin and breast skin by all treatments. Therefore, rather than sharing similarity with Th2 cells, the cytokine profile of skin MC at baseline resembles that of monocytes. Of note, MC precultured in the presence of IL-4 [alone or plus stem cell factor (SCF)] before anti-IgE stimulation, acquired the ability to produce IL-5, and IL-1 β was concomitantly suppressed. Additionally, strong up-regulation of IL-6 by SCF was observed, which was inhibited by IL-4. In summary, we present a detailed analysis of the cytokine array of human skin MC immediately upon isolation; demonstrate that MC from different skin compartments, although producing the same pattern of cytokines, display quantitative differences in several aspects; and provide further evidence that MC possess a proinflammatory capacity, which can, however, be altered by microenvironmental stimuli, substantiating the marked plasticity of the cells. *J. Leukoc. Biol.* 75: 244–252; 2004.

Key Words: interleukin · innate immunity · inflammation · cell plasticity

INTRODUCTION

Mast cells (MC) are specialized immune cells that occupy an important position in immediate hypersensitivity reactions and

late responses to allergens (reviewed in refs. [1, 2]). Additionally, there is growing evidence that these cells are involved in other processes such as wound repair, tissue remodeling, angiogenesis, and in particular, innate-immune responses to bacterial and parasitic infections [3–7]. Therefore, other than their detrimental role in allergic inflammation, MC likely contribute to processes that are beneficial to the host, acting as significant constituents in the complex network against intruding pathogens. By the strategic localization adjacent to blood and lymph vessels in many tissues, MC seem ideally suited to perform key functions in the regulation of vasoactive processes and the control of leukocyte infiltration into tissues through the production of cytokines and other mediators [2, 8, 9].

Studies about the cytokine expression by MC have in the past focused primarily on rodent systems, which differ in many respects from their human counterparts [2]. There are fewer reports on cytokine patterns of MC from human tissues, and as these rely in general on immunohistochemical stainings, quantitative data are often lacking. However, the development of techniques to purify MC to near homogeneity and with high yield allowed us to address these points directly and in detail. Recent systematic studies have discerned the cytokine profile of intestinal MC, which belong to a typical mucosal MC subcategory. These studies have shown that a variety of mediators can in principle be produced by MC, with the prerequisites for the different cytokine subtypes being, however, clearly distinct [10, 11]. In particular, the “classical” MC activation route via high-affinity immunoglobulin (Ig)E receptor affected the production of only some mediators but obviously did not impact others, such as interleukin (IL)-6 and IL-8 [10]. In addition, typical proinflammatory cytokines were found to be a characteristic feature of these cells, and only long-term pretreatment with IL-4 and stem cell factor (SCF) induced several T helper cell type 2 (Th2) cytokines [10, 11].

By their constitutively high expression of MC chymase, skin MC are classified as representative, connective tissue MC, which can be distinguished from mucosal MC that express only tryptase and represent the dominant MC subtype in the gut or

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lung [12]. Other than differences in the composition of neutral proteases, MC heterogeneity also extends to phenotype, ultrastructure, secretagogues that elicit MC degranulation, and the pattern of biologically active compounds produced following activation [1, 13]. Using MC purified from breast skin, a recent study reported that skin MC are producers of tumor necrosis factor α (TNF- α) and IL-8 (with low preformed levels of both mediators being found) but obviously not of IL-4, IL-5, or IL-13 [14]. As skin is a heterogeneous organ, with very different microenvironments prevailing in skin at different locations, we sought in the present study to clarify in depth the cytokine profile produced by MC from two distinct skin compartments (breast skin and foreskin) in direct comparison and under several activation protocols. In addition, the relationship of blood-derived monocytes and immature human leukemic MC (HMC-1 5C6) was also investigated. Finally, the consequences of different long-term priming protocols on the profile of MC-generated cytokines were assessed.

Our results imply that MC in the skin do not directly contribute to processes specifically linked to a Th2 cytokine response under nonpathological or baseline conditions. On the contrary, by the striking resemblance to monocytes/macrophages and their strategic position within tissues, MC may be of primary relevance to the establishment of innate immunity. The marked plasticity of skin MC is however underlined by different long-term priming protocols under which the cells acquire the ability to release a selective panel of Th2-associated cytokines and concomitantly decreasing their proinflammatory function.

MATERIALS AND METHODS

Reagents

Phorbol 12-myristate-13-acetate (PMA) was purchased from Sigma (Deisenhofen, Germany). The calcium ionophore A23187, bacterial lipopolysaccharide (LPS), phytohemagglutinin (PHA), and mouse IgM-antihuman IgE Fc fragment (with a protein concentration of 2 mg/ml) were supplied by Calbiochem-Novabiochem (Bad Soden, Germany; Cat. No. 411500). This mouse antihuman IgE is highly specific for the Fc portion of human IgE and does not cross-react with any other human Ig subclass. In addition, the ability of this antibody to activate skin MC is solely attributed to its affinity for human IgE, as neither an irrelevant mouse-IgM control antibody nor complete mouse serum is able to degranulate skin MC, as assessed in preceding tests (values of histamine release in percent of complete, assessed as described below: spontaneous, 3.2 ± 0.6 ; mouse IgM-antihuman IgE Fc fragment, 17.3 ± 5.1 ; irrelevant mouse-IgM control, 2.9 ± 0.4 ; complete mouse serum, 2.9 ± 0.3 ; $n=4$).

Cell lines and purification of primary cells

Human skin MC were isolated according to a published protocol [15] with several modifications using macroscopically normal juvenile foreskin or adult breast skin. Typically, several foreskins were combined and used for one experiment, and in the case of breast skin, material from one single patient was used per preparation. The skin was cut into stripes and treated with dispase (Boehringer-Mannheim, Mannheim, Germany) at 0.5 mg/ml and 4°C overnight. Thereafter, the epidermis was removed from the dermis, the latter chopped in small pieces and digested with collagenase at 10 mg/ml (type 4, Worthington, Lakewood, NJ) for 1 h at 37°C, after which time isolated cells were separated from remaining tissue by three steps of filtration (pore sizes 250, 100, and 40 μ m). In the case of breast skin (derived from healthy women undergoing breast-reduction surgery), hyaluronidase at 5 mg/ml (type 1S, Sigma), DNase I at 10 μ g/ml (Roche, Basel, Switzerland), and 5 mM MgCl₂ were present in

addition to collagenase, and a second digestion step at 37°C was applied. After tissue digestion, foreskin cell dispersates typically contained $0.7\text{--}1.1 \times 10^6$ MC/g skin tissue with an initial purity of 2–3%, and breast skin dispersates, as a result of the much lower cell density in the dermis, only contained approximately 0.1×10^6 MC/g skin tissue but with substantially higher initial purity of approximately 10%. MC were further purified from these suspensions by positive selection using anti-c-kit monoclonal antibody YB5.B8 (kindly provided by Dr. L. K. Ashman), goat antimouse Ig-coated magnetic beads (Miltenyi Biotec, Bergisch Gladbach, Germany), and a magnetic cell sorter separation device. MC purity in these preparations typically exceeded 95%, as assessed by acidic toluidine blue staining (0.1% in 0.5 N HCl). Final yield was 60–80% (breast skin) and 90% (foreskin), respectively.

Skin MC were cultured in RPMI medium, supplemented with 10% heat-inactivated fetal calf serum (FCS), 4 mM L-glutamine, and antibiotics (all from Seromed, Berlin, Germany) and were processed for downstream applications, as described below.

Monocytes were isolated from heparinized whole blood drawn from healthy volunteers. Following density gradient centrifugation over Histopaque (Sigma), monocytes were purified from the peripheral blood mononuclear cell (PBMC) fraction by negative selection with magnetic beads (Dyna, Hamburg, Germany), according to the supplier's protocol. Monocytes obtained by this method were very uniform, showing a distinct peak on cell counting (Casy 1, cell counter and analyzer, Schärfe System, Reutlingen, Germany) and were ~95% positive for CD14. In selected cases, monocytes were isolated by adhesion, as described [16]. The same medium as for skin MC was used.

For comparison with skin MC, subclone 5C6 [17], raised from a human leukemic MC line (HMC-1, kindly provided by J. H. Butterfield et al. [18]) was used. Cells were routinely grown in basal Iscove's medium, supplemented with 10% FCS, L-glutamine, and antibiotics as above and 10^{-5} M monothioglycerol (Sigma). For supernatant collection, cells were washed in the same medium but without FCS, and stimulations were performed under serum-free conditions.

The media used for routine culture of the different cell types are referred to as standard media hereafter.

Cell treatment and sample collection

All cell types used in the study were plated in culture dishes at 1×10^6 /ml in standard medium and kept at 37°C for 24 h. Skin MC were left untreated or stimulated with mouse-antihuman IgE (dilution 1:20,000) or with PMA (at 25 ng/ml). In certain tests, PMA was combined with the calcium ionophore A23187 (at 500 nM). Whenever indicated, stimulations were performed under serum-free conditions. After incubation, supernatants were removed, remaining cells and debris pelleted, and aliquotted cell-free supernatants kept at -80°C until assaying for cytokine concentration. Remaining cells were lysed at 1×10^6 /ml in lysis buffer (1% Triton X-100, 50 mM Tris-HCl, pH 8.0, 150 mM NaCl, 100 μ g/ml phenylmethylsulfonyl fluoride, 1 μ g/ml aprotinin, and 1 μ g/ml leupeptin) for 30 min on ice and centrifuged at 20,000 g, and the supernatants thereof were used for quantitation of cytokines by enzyme-linked immunosorbent assay (ELISA).

Monocytes were kept in medium only or were stimulated with LPS (at 100 ng/ml), and HMC-1 5C6 cells were stimulated with PMA, as above. Supernatants from the different cells were tested in parallel in ELISA tests.

Long-term culture of skin MC

For priming experiments, human skin MC were split into three parts after purification, each cultured in standard medium at an initial density of 5×10^5 /ml with the following cytokines: IL-4 (20 ng/ml), SCF (100 ng/ml), or IL-4 + SCF (purchased from R&D Systems, Wiesbaden, Germany). Semidepletions of the culture medium with the addition of cytokines were done twice weekly. After 2 weeks, cells were counted, and viability was assessed by trypan blue exclusion. Cells were then replated at a density of 1×10^6 /ml and primed with IgE (at 5 μ g/ml overnight), washed, and then stimulated with anti-IgE (1:20,000, as above). ELISA investigated the supernatants of these stimulations for cytokine content. In certain experiments, MC were first cultured in the presence of IL-4 + SCF for 2 weeks as above, washed, and split into two parts, one of which was continuously supplied with both cytokines, and the other received SCF only to test for the effects of IL-4 withdrawal.

Histamine release

Cell suspensions were divided into aliquots, washed twice with piperazine-N,N'-bis[2-ethanesulfonic acid]-albumin-glucose buffer containing 3 mM

CaCl₂ and 1.5 mM MgCl₂, pH 7.4 (PAG-CM), resuspended at 4×10^5 cells/ml, and challenged for 30 min at 37°C with anti-IgE (1:20,000) or kept in buffer only (PAG-CM) for spontaneous release, and supernatants were stored at -20°C until measurement. Total cellular histamine content was assessed upon cell lysis with 1% perchloric acid. Histamine amounts were determined by an automated fluorescence method [19], using an autoanalyzer (Borgwald Technik, Germany, Hamburg).

ELISA

Concentrations of IL-1 β , IL-4, IL-10, and TNF- α in the supernatants were determined by using high-sensitivity ELISA (all from R&D Systems), and (as a result of unavailability of kits with enhanced sensitivity or the presence of sufficient amounts of cytokines, which easily allowed detection) standard kits were used for IL-3, IL-5, IL-6, IL-8, IL-13, IL-16, and IL-18 (R&D Systems), as detailed by the supplier. In the case of IL-5, incubations were done overnight to enhance sensitivity, as recommended by the supplier. To detect IL-3, IL-4, IL-5, IL-10, and IL-13, undiluted or 1:2 diluted supernatants and lysates were used, and the other samples were diluted appropriately to ensure detection in about the central part of the standard curve. Cell-free culture media served for control purposes. No cross-reactivity of FCS or media components with any of the cytokine kits used could be detected.

Reverse transcriptase-polymerase chain reaction (RT-PCR) assay

Total cellular RNA was isolated using the RNeasy total RNA kit, digested with RNase-free DNase (Qiagen, Hilden, Germany) and quantitated using the RiboGreen RNA quantitation kit (Molecular Probes, Leiden, The Netherlands). Total RNA (~300 ng per 20 μ l reaction volume) was reverse-transcribed with a first-strand synthesis kit (Boehringer-Mannheim), using random priming, as detailed by the manufacturer, and aliquots of the reaction product were mixed with specific primers and a PCR master mix (Boehringer-Mannheim) containing all components required for PCR amplifications. Optimum conditions such as annealing temperatures (62°C for IL-4, IL-5, and IL-13; 66°C for TNF- α ; and 68°C for IL-8) and cycle numbers were determined in preliminary tests. In the case of IL-4, the maximum amount of cDNA and cycles (40) was applied. Primers were used at 500 nM each and have been described elsewhere [10]. As a positive control, PBMCs stimulated for 4 and 24 h, respectively, with PHA at 2 μ g/ml were used.

Statistics

The statistical significance of differences between values was assessed by nonparametric Mann-Whitney test (two-tailed, unpaired) when two columns (foreskin vs. breast skin MC) were compared. In the case of more than two treatment groups (medium, PMA, anti-IgE), statistical analyses were performed by Kruskal-Wallis test with post-test for unpaired samples.

RESULTS

Comparative cytokine release by human foreskin and breast skin MC—evidence for a proinflammatory function at baseline

To define in detail the cytokine production by human skin MC, we used two individual MC subtypes, i.e., MC purified from juvenile foreskin in comparison with adult breast skin. These two MC subsets do not exhibit morphological differences and display about the same responsiveness toward anti-IgE-mediated release of preformed mediators such as histamine (**Fig. 1**). The capacity of both skin MC types to produce a wide range of cytokine mediators (which are not or are only slightly preformed) was determined in the absence of stimuli or following stimulation by anti-IgE or PMA. The results are summarized in **Table 1**. Although absolute concentrations varied from cell preparation to cell preparation (see the ranges given in Table

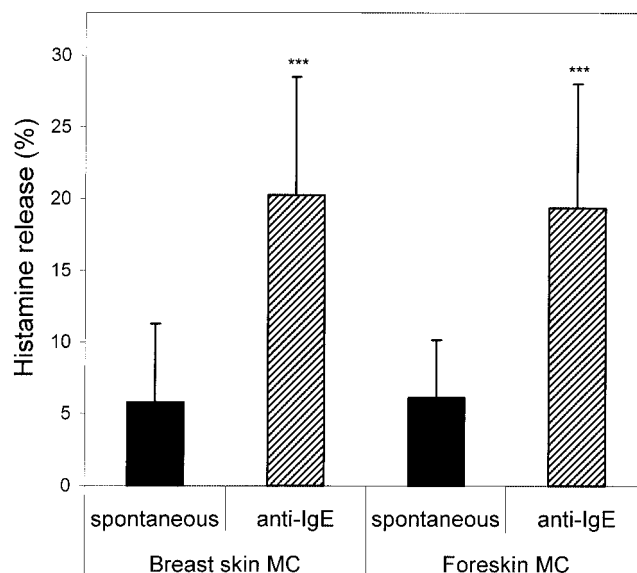


Fig. 1. Comparative histamine release of foreskin and breast skin MC. Upon isolation, the two skin MC subtypes were triggered by anti-IgE, and histamine release was measured, as described in Materials and Methods. Data are expressed in percent of total histamine content and are the mean SD of 20 (breast skin) and 18 (foreskin) independent assays. ***, $P < 0.001$, versus spontaneous release. No significant differences were found for MC from the two skin compartments.

1), the tendency within one given preparation was similar with regard to the different treatments.

The following conclusions can be drawn: Of the various mediators tested, only IL-1 β , IL-6, IL-8, and TNF- α were secreted in sufficient amounts to allow for clear detection. Conversely, a broad range of cytokines previously demonstrated to be expressed by certain MC subtypes, including IL-3, IL-4, IL-5, IL-10, and IL-13, remained undetectable in MC from both skin sites, irrespective of activation. Low levels of IL-18 secretion were detected in skin MC and HMC-1 5C6 cells (and both contained much higher IL-18 levels in the lysates; see below, and data not shown). Assaying for IL-16 again yielded negative results. Taken together, a typically proinflammatory cytokine array (rather than a Th2 profile) is thus characteristic of human MC in the skin, which covers the most significant constituents, i.e., IL-1 β , IL-8, IL-18, and TNF- α [20–23].

Cytokine production by skin MC also occurred in the absence of an additional stimulus, in accordance with data on intestinal MC [10]. Although PMA was found to enhance this baseline production very potently for most proinflammatory mediators, Ig Fc ϵ receptor 1 (Fc ϵ RI) cross-linking was only active in inducing TNF- α , corresponding with the results at the mRNA level (**Fig. 2**). This is again largely in line with data on mucosal MC [10]. It is interesting that IL-6 was not affected by any treatment, suggesting different prerequisites of signaling and/or transcriptional regulation underlying its expression [24, 25]. Of note, IL-8 and TNF- α were synergistically enhanced when PMA was combined with calcium ionophore (not shown), but the addition of ionophore was suppressive with regard to IL-1 β , where PMA-triggered release dropped to ~50% under combined treatment (not shown).

TABLE 1. Cytokine Profiles of Human Foreskin and Breast Skin MC

Cytokine		Foreskin MC			Breast skin MC			HMC-1	Monocyte ^b
		Medium	Anti-IgE	PMA	Medium	Anti-IgE	PMA	5C6 ^a	
IL-1 β	mean	130 (8)	179 (9)	392 (7)*	25 (3) [#]	36 (3) [#]	164 (3)	41 (7)	3104 (4)
	range	45–253	45–421	116–812	22–28	4–63	100–211	23–59	1042–4239
IL-3	mean	0 (2)	0 (2)	0 (2)	0 (3)	0 (3)	0 (3)	0 (3)	0 (4)
	range								
IL-4	mean	0 (2)	0 (2)	0 (2)	0 (3)	0 (3)	0 (3)	3.9 (8)	0 (4)
	range							2.6–6.7	
IL-5	mean	0 (2)	0 (2)	0 (2)	0 (3)	0 (3)	0 (3)	0 (3)	0 (4)
	range								
IL-6	mean	627 (7)	625 (7)	509 (5)	71.6 (9) ^{###}	35.5 (6) ^{####}	n.d.	56 (10)	9743 (4)
	range	203–1730	172–1867	92–1578	17.5–146.5	11.4–80.2		17–189	760–15800
IL-8	mean	3.5 (8)	3.9 (7)	22.8 (10)*	4.1 (13)	5.0 (9)	n.d.	18.6 (26)	277.0 (9)
	range	0.8–14.2	0.4–15	2.1–63	1.2–11.6	1.3–12.7		2.4–42.1	74–597
IL-10	mean	0 (2)	0 (2)	0 (2)	0 (3)	0 (3)	0.9 (3)	0 (2)	931 (4)
	range						0–1.6		227–1733
IL-13	mean	0 (2)	0 (2)	0 (2)	0 (3)	0 (3)	0 (3)	0 (4)	0 (4)
	range								
IL-16	mean	0 (3)	0 (3)	0 (2)	0 (3)	0 (3)	0 (2)	0 (2)	64 (3)
	range								53–76
IL-18	mean	34.3 (3)	33.9 (3)	31.0 (5)	29.0 (3)	31.6 (3)	32.5 (3)	1320 (3)	504 (6)
	range	17.3–62	16–67	14–59	26.5–33.7	26.7–41.2	26.9–43.6	1249–1348	128–1348
TNF- α	mean	10.6 (5)	91.7 (7)	285 (9)***	8.2 (12)	127.6 (6)***	n.d.	1820 (9)	269 (7)
	range	5–23	12–270	79–631	3.2–12.8	23.5–305.6		379–3885	148–640

Upon purification, as described in Materials and Methods, skin mast cells (at $1 \times 10^6/\text{ml}$) were kept in medium only or stimulated by anti-IgE or PMA for 24 h at 37°C. Data are expressed as the mean in pg/ml (ng/ml for IL-8 only). The number of independent tests (corresponding to individual mast cell preparations) is given in parentheses. n.d., Not done; 0, cytokine concentration was below the detection limit of the ELISA test used (standard ELISA kits were used for: IL-3, IL-5, IL-6, IL-8, IL-13, IL-16, and IL-18; high-sensitivity ELISA kits were used to detect: IL-1 β , IL-4, IL-10, and TNF- α). ^a Cells were cultured under serum-free conditions in the presence of PMA (25 ng/ml). ^b Cells were cultured in standard growth medium for 24 h in the absence of additional stimuli. * $P < 0.05$; *** $P < 0.001$ versus the medium control (Kruskal-Wallis test). [#] $P < 0.05$; ^{###} $P < 0.001$; ^{####} $P < 0.01$; versus foreskin MC (Mann-Whitney test).

No qualitative differences in the cytokine pattern were found between juvenile foreskin and adult breast skin MC. On a quantitative basis, however, breast skin MC produced substantially lower levels of IL-1 β and IL-6, as compared with foreskin MC, and no difference was detected for IL-8 and TNF- α , which showed overall highest variability among preparations.

The cytokine pattern of immature HMC-1 5C6 cells strikingly resembled that of skin MC with the exception that HMC-1 5C6 produced in addition to low levels of IL-4, possibly hinting at the capacity of MC to produce this cytokine at a less-differentiated state. IL-4 is probably the most critical cytokine of the Th2 subset based on the fact that it is not only a distinguishing marker between Th1 and Th2 (i.e., interferon- γ -vs. IL-4-producing) cells but also in that it is the cytokine itself that is required during antigen presentation to establish Th2 progeny. Our systematic analysis, together with a previous study [14], demonstrates that at this stage of differentiation, MC in nonlesional skin do not produce IL-4 under standard activation protocols. This quality may be confined to less-mature MC, MC in the respiratory tract, or MC under pathological conditions [26–28].

There was a particularly striking resemblance in the array of cytokines between skin MC and monocytes. One distinguishing factor was IL-10, however, whose production was obviously not induced in the former but as expected, was well detectable in the supernatants of monocytes.

Similar results as for supernatants were obtained when MC lysates (performed 24 h after treatment) were assayed for

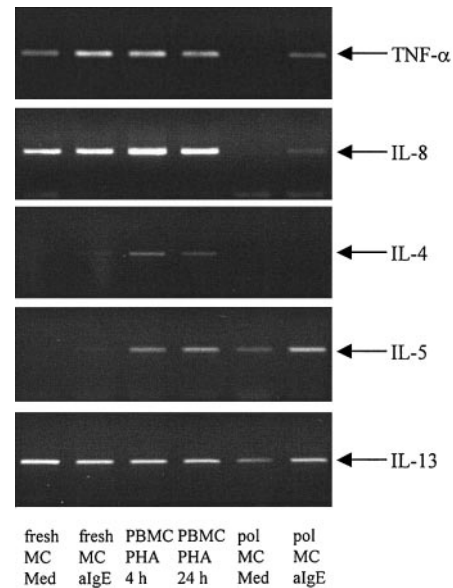


Fig. 2. Transcript levels of cytokine genes in freshly isolated and SCF + IL-4-polarized breast skin MC before and following cross-linking of Fc ϵ RI. RT-PCR was run, as described in Materials and Methods. Fresh MC Med, MC immediately after isolation and then kept in medium for 4 h; fresh MC algE, MC immediately after isolation and then stimulated by anti-IgE for 4 h; PBL PHA 4 h/24 h, PBMC stimulated with PHA for 4 or 24 h, respectively (positive control); pol MC Med, MC polarized by SCF + IL-4 for 2 weeks and then kept in medium for 4 h; pol MC algE, MC polarized by SCF + IL-4 for 2 weeks and then stimulated by anti-IgE for 4 h.

TABLE 2. Lysate-Associated Mast-Cell Cytokines after 24 h

Cytokine		Medium		Anti-IgE		PMA	
		Supernatant	Lysate	Supernatant	Lysate	Supernatant	Lysate
IL-1 β	mean	125	19	168	21	458	752
	range	45–220	8–31	45–421	12–30	123–650	457–1223
IL-3		0	0	0	0	0	0
IL-4		0	0	0	0	0	0
IL-5		0	0	0	0	0	0
IL-6	mean	770	168	691	127	878	218
	range	104–1730	59–278	86–1564	53–203	220–1834	121–378
IL-8	mean	3.4	1.6	3.9	1.6	19.8	10.2
	range	1–14.2	0.22–3.0	0.9–15.0	0.28–2.6	2.2–63.0	4.8–17.4
IL-10	mean	0	0.8	0	1.0	0	1.1
	range	0	0–2.4	0	0–3.0	0	0–3.3
IL-13		0	0	0	0	0	0
IL-18	mean	34.3	291	33.9	227	31.0	187
	range	17–62	101–700	16–67	171–296	14–59	170–203
TNF- α	mean	7	0.3	22	2.4	301	79
	range	2.1–12.3	0–1.0	6.9–41.2	0–5.4	79–835	31–161

Foreskin (IL-1 β , IL-6, IL-8, IL-18, and TNF- α) or breast skin (the other cytokines) MC were challenged, as described in Table 1 for 24 h, and supernatants and lysates were tested for cytokine concentration in direct comparison. Results are expressed in pg (or ng)/ml/10⁶ cells and are the mean of three to six individual tests, with the ranges of values given below.

cytokine concentration. In particular, no Th2 cytokines were found, substantiating that their absence in supernatants was not a result of defective and/or retarded secretion (**Table 2**). Additional evidence came from the virtual absence of IL-4- and IL-5-specific transcripts in freshly isolated MC by the RT-PCR technique, and unexpectedly, IL-13-specific mRNA was present regardless of stimulation (Fig. 2).

Nevertheless, the comparison between supernatants and lysates uncovered some interesting differences among mediators. In general, the lysates contained lower levels of remaining cytokines as compared with the amounts accumulated in the supernatants over a period of 24 h (Table 2). One exception was PMA-induced IL-1 β , which is known to be produced as an inactive precursor in the cytoplasm by an unconventional pathway, requiring cleavage by caspase 1 for conversion into the active form before release [20]. This additional level of regulation may explain why the remaining amounts in the cell after 24 h are fairly high. This also holds for IL-18, of which

higher levels were detected in MC lysates, most likely representing the inactive, preformed precursor (Table 2).

Effects of serum on skin MC cytokine production

The results given in Table 1 are based on standard conditions using serum-containing culture media. To test if the relatively high baseline release of cytokines was partially affected by serum components, we next compared cytokine levels in the presence or absence of serum. Serum indeed may have diverse effects on cytokine production and/or possibility of detection, based on activatory or inhibitory factors acting on the cells or stabilizing versus degrading factors acting on the cytokines, respectively.

As shown in **Table 3**, mediator levels were in general, 50–80% reduced under serum-free versus serum-containing conditions, possibly as a result of decreased activator protein-1 activity under serum deprivation [29], but the differences between treatments (medium, anti-IgE, PMA) were similar in both cases. There were two prominent exceptions, however.

TABLE 3. Effects of Serum on MC Cytokine Release

Cytokine		Medium		Anti-IgE		PMA	
		Serum	Serum-free	Serum	Serum-free	Serum	Serum-free
IL-1 β	mean	96	48	86	50	503	63
	range	45–128	30–67	45–132	35–80	116–813	31–120
IL-6	mean	770	251	787	325	782	220
	range	104–1730	39–469	86–1867	33–613	220–1578	78–369
IL-8	mean	3.7	0.9	3.8	1.0	21.5	4.9
	range	0.9–14.2	0.3–1.6	0.5–15	0.5–1.6	2.1–62.4	3.7–6.1
TNF- α	mean	7.2	10.5	15.8	38.4	330	260
	range	1.8–12.3	8.3–12.1	6.7–28.0	23.9–59.4	117–530	73–601

Foreskin MC were challenged as described in Table 1 for 24 h in the presence or absence of 10% FCS, and supernatants were tested for cytokine concentration in direct comparison. Results are expressed in pg (or ng)/ml/10⁶ cells and are the mean of three to six individual tests, with the ranges among preparations given below.

The first concerns IL-1 β , which was strongly induced by PMA (resulting in a fivefold to sixfold increase if compared with the medium control) only when serum was present in the culture medium, and the inducing effect was not seen in the absence of serum. Analysis of the corresponding lysates under both conditions indicated that the low levels in serum-free supernatants resulted primarily from impaired secretion rather than production (data not shown). The other exception was TNF- α for which higher amounts were found under serum-free conditions following Fc ϵ RI cross-bridging.

Effects of long-term administration of IL-4 and SCF on skin MC cytokine production

Long-term culture in the presence of SCF + IL-4 (but not SCF alone) *in vitro* has been reported to render intestinal human MC capable of producing IL-3, -5, and -13, thereby switching them toward a typical Th2 profile [10, 11]. We also tested an analogous strategy for human skin MC, but in addition, IL-4 alone was applied as a further treatment.

In preliminary experiments, it was found that the survival rate of skin MC in medium only, *i.e.*, without the addition of exogenous cytokines, is poor after 2 weeks (with $\sim$ 15% of the cell number originally seeded being recovered with most of the remaining cells displaying signs of cell death). However, IL-4 alone was able to significantly enhance MC survival (to $39.9 \pm 11.5\%$, $n=12$), although less efficiently than SCF ($53.7 \pm 15.9\%$, $n=12$), and the combination of both was superior to either mediator alone regarding MC recovery ($89.2 \pm 21.4\%$, $n=12$). As determined by trypan blue exclusion, the viability of the recovered cells always exceeded 98%, irrespective of the type of pretreatment. One interesting and unexpected result was therefore the identification of IL-4 as a survival-prolonging factor of human skin MC.

It is interesting that skin MC obviously acquired the ability to produce IL-5 and occasionally, IL-10 in the presence of IL-4 (with or without the addition of SCF, **Table 4**), and IL-13 and IL-3, determined for three and one preparations, respectively, remained undetectable (not shown). On comparison with mucosal MC [10, 11], the panel of Th2 cytokines inducible by IL-4 pretreatment seems more restricted in the case of connective tissue MC in human skin. Nevertheless, as IL-4 alone

rendered skin MC capable of producing IL-5 and (less frequently) IL-10 in response to anti-IgE, synergy with SCF was unlikely needed. The amounts of IL-5 secreted in the absence of Fc ϵ RI cross-linking were five- to tenfold lower than in cells stimulated by anti-IgE (data not shown), thereby further confirming the IL-5 gene (in addition to TNF- α) as a downstream target of the signaling cascade via the IgE receptor. The up-regulation of IL-5 following Fc ϵ RI cross-linking of polarized MC was confirmed at the message level, and IL-4 expression remained undetectable irrespective of pretreatment (Fig. 2). As with freshly isolated MC, IL-13 transcript was found in polarized MC, where it was further up-regulated by signaling through the IgE receptor (Fig. 2), although this mRNA expression again did not result in the generation of detectable levels of the mature IL-13 protein.

Also of interest, skin MC cultured in IL-4 completely lost the ability to produce IL-1 β , thereby reducing their inflammatory potential concomitantly with the acquisition of a Th2 repertoire, but long-term culture by itself was apparently suppressive as for IL-1 β production (Tables 1 and 4). Conversely, cells cultured in the presence of SCF alone had a strongly enhanced capacity of IL-6 production compared with freshly isolated cells (446 vs. 35.5 pg/ml; Tables 1 and 4), and the presence of IL-4 almost completely abolished this inducing effect from SCF. In addition, although TNF- α production was also reduced with all long-term culture protocols if compared with MC upon isolation (Tables 1 and 4; Fig. 2), the differences were substantially lower than those detected with IL-1 β . Moreover, although cells cultured in IL-4 alone were compromised for TNF- α production, IL-4 had no negative effect when combined with SCF. This is in sharp contrast to the inhibitory effect from IL-4 on IL-1 β and even more so, on IL-6 production (Table 4).

Upon withdrawal of IL-4 from long-term cultures for a further week, the capacity of MC to produce IL-5 was reversed again, and levels of IL-1 β increased concomitantly (data not shown), suggesting that the changes induced by IL-4 are reversible and require the constant supply with the cytokine.

Taken together, the marked plasticity of the mature tissue MC, in the intestine [10, 11, 30] and in the skin (as shown in this study), is of considerable note.

TABLE 4. Effect of Long-Term Priming by IL-4 Alone, SCF Alone, or IL-4 + SCF on Skin MC Cytokine Pattern Elicited by Anti-IgE

Cytokine		Foreskin MC			Breast skin MC		
		IL-4	SCF	SCF + IL-4	IL-4	SCF	SCF + IL-4
IL-1 β	mean	0 (4)	8.2 (4)	3.1 (4)	0 (3)	6.1 (3)	3.0 (4)
	range	0	0–12.9	0–5.2	0	5.3–6.8	1.6–4.8
IL-5	mean	73 (5)	3.5 (5)	115 (5)	24.1 (3)	2.9 (3)	76.1 (5)
	range	12–113	0–17.3	19–280	4.8–41	0–8.7	57–103
IL-6	mean	n.d.	n.d.	n.d.	21.3 (3)	446 (3)	55.1 (3)
	range				10.8–36.1	295–638	41.8–72.3
IL-10	mean	31 (3)	0 (3)	48.7 (3)	0 (2)	0 (2)	0 (2)
	range	0–90	0	0–101	0	0	0
TNF- α	mean	6.2 (3)	40.8 (3)	66.3 (3)	2.1 (4)	53.5 (4)	61.9 (4)
	range	3.9–8.4	4.4–77	52–74	0–4.0	18–91	15–98

Data are expressed as pg/ml. n.d., Not done.

DISCUSSION

In view of the well-known heterogeneity of MC, the response of the cells to IgE and non-IgE-mediated stimulation will critically depend on the anatomic location. In the present study, we used typical connective tissue-type MC isolated from two skin compartments for direct comparison and investigated in detail the cytokines produced following different treatments. It was found that upon isolation from skin tissue, MC are unable to produce several mediators previously ascribed to the lineage, with the experiments in the past, however, having been biased toward rodent systems and/or the detection only by immunohistochemical techniques. Our study, therefore, revealed several rather unexpected findings. In particular, IL-4, IL-5, IL-10, and IL-13 were not found in the supernatants of breast skin or foreskin MC immediately upon isolation from tissue, and the same negative results were obtained for the respective lysates, suggesting the true absence of these mediators in MC from normal skin. The absence of IL-4 and -5 was corroborated by negative results also at the mRNA level, and there was some discrepancy in this regard with IL-13, for which fairly high levels of mRNA were detected, and the protein was lacking. The reason for this inconsistency is unknown at present, but as the baseline expression of this transcript in the absence of stimulation was high in freshly isolated MC, it cannot be excluded that the gene is transcribed continuously in skin MC without being translated. Taken together, MC in normal skin do not appear important producers of typical Th2 cytokines but rather produce a cytokine profile strongly reminiscent of (classically activated) macrophages with a clear dominance of proinflammatory mediators, i.e., IL-1 β , IL-8, and TNF- α . IL-6, possessing pro- and anti-inflammatory as well as growth-stimulating characteristics [22], were also found to be produced constitutively with no regulation by PMA or anti-IgE (but regulated in a positive manner by SCF but not SCF+IL-4 or IL-4 alone, Table 4). IL-18, a cytokine highly implicated in repelling mechanisms toward bacterial infections [23], was also found at low levels in supernatants (and much higher in lysates). The proinflammatory cytokine pattern thus seems quite universal among MC of human origin, given the similar results reported for intestinal MC, the latter, however, not having been investigated directly upon purification [10, 11]. With selected cytokines (IL-1 β , IL-6), quantitative differences between foreskin and breast skin MC were found. Possible reasons for this may encompass the age or the sex of the donors as well as the precise anatomic location of the skin compartment. Definite clarification of this aspect will require the development of techniques that allow for the accurate quantification of cytokines when using extremely low cell numbers that can, e.g., be isolated from routine skin biopsies. However, the slight but significant quantitative differences among skin MC subsets that are shown here are of potential interest and demonstrate that the well-known MC heterogeneity does even extend to a certain degree to the different parts of the very same organ.

In general, production and secretion of these cytokines were interconnected processes, as differences among treatments (medium only, anti-IgE, PMA) were quite similar for lysates and supernatants. This indicates that the majority of mediators is probably released by a default pathway, once their produc-

tion has been initiated. This does, however, not completely apply to IL-1 β and IL-18, both of which are produced in the cytoplasm by an uncommon mechanism and require cleavage by caspase 1 before release [20].

In an attempt to discern the reason for the relatively high, spontaneous secretion of proinflammatory cytokines, the presence of serum was identified as one (but obviously not the only) inducing factor. The exception of higher levels of TNF- α under serum-free conditions may, conversely, be a result of its enhanced stability in the absence of serum-derived proteases, which is corroborated by the short half-life of this cytokine in biological fluids and a strong decrease of TNF- α in serum-containing versus serum-free supernatants of HMC-1 5C6 cells (data not shown).

A further, noteworthy observation was that in freshly isolated cells, only TNF- α was positively regulated by IgE receptor cross-linking, demonstrating that this classical MC activation protocol leads to a selective rather than overall cytokine response. Conversely, PMA [as a well-known nuclear factor (NF)- κ B activator whose function is mandatory to the regulation of proinflammatory genes; ref. 31] was found to up-modulate IL-1 β , IL-8, and TNF- α , showing that the former, although resistant toward anti-IgE-mediated stimulation, can in principle be further elevated in MC. Whether PMA mimics specific bacterial (or parasitic) products is unknown at present but appears an interesting hypothesis in view of the growing evidence for MC as significant participants in the establishment of innate immunity [3–7]. Conversely, the signaling cascade initiated through the Fc ϵ RI is thought to be more complex, with numerous kinases being activated in a timely, coordinated manner. The most obvious distinction from PMA, however, is the immediate Ca²⁺ signal and thus, among others, the activation of the calcineurin NF-activated T cell cascade. Although our general understanding of the precise patterns of transcription factors that need to combine on the regulatory elements of each individual cytokine gene, acting in a positive or in a negative manner, is still rather limited (and this holds even more for the MC), it is of note that certain cytokines (such as IL-1 β) appear to be inhibited rather than further enhanced by Ca²⁺ in MC, as opposed to other mediators such as TNF- α (data not shown). This is further evidence that the events driving expression of each individual cytokine gene bear overlapping but also divergent components. Distinct prerequisites for the expression of each cytokine gene can also be inferred from our data with the different long-term priming protocols. These selective patterns of reactivity likely endow the cell with the required flexibility to respond to environmental factors with the production of only those mediators that appear beneficial to the host at that particular time (but may be detrimental in cases of dysregulation, which is frequently encountered under pathological conditions).

Although delineation of the molecular mechanisms driving transcription of each individual cytokine gene will thus require numerous future efforts, the *in vivo* importance of MC-derived cytokines such as TNF- α in immune responses toward certain infections is indeed well appreciated [32–34], and further data supporting a critical role of MC-derived cytokines (in particular, TNF- α and IL-8) for acute infections and neutrophil recruitment have been presented recently [35, 36]. In addition,

human MC generated in culture from progenitor cells have been demonstrated to express several members of the Toll-like receptor (TLR) family, although their pattern seems to depend on the protocol used for MC generation [37, 38]. Although the repertoire and functionality of TLRs thus need confirmation for MC that have completed maturation in situ, the above scenario may well apply also to human tissues including the skin: Following infection and other types of insult, MC-mediated neutrophil recruitment and activation would occur by TNF- α and IL-8 secretion, acting in concert on endothelial cells in the vicinity of MC (by up-regulating critical adhesion molecules) and on neutrophils (by supplying a chemotactic and activating stimulus, respectively). This MC function as an initiator of innate immunity to pathogens may be further amplified by cytokines such as IL-1 β and IL-18. Although these latter cytokines have been less well investigated in the context of the MC, IL-1 β has been recently shown to be induced by bacterial products in MC generated from hematopoietic precursors [37].

One striking feature of the study was the similarity between (freshly isolated) skin MC and monocytes, with the majority of mediators being produced or not produced by both myelocytes. One exception was the obvious absence of IL-10 production, a cytokine normally secreted by macrophages with delayed kinetics to dampen ongoing inflammation after the tissue insult has been resolved. Therefore, MC-initiated inflammation may be more difficult to clear, as no autocrine negative-feedback loop is apparent from our data. Nevertheless, the similarity of cytokine patterns between MC and macrophages further substantiates their common origin [39] and implies that depending on the tissue, MC may amplify the actions of resident and elicited macrophages or be even the most relevant cells in this regard, given their much higher density at several sites [40]. Even the lower levels of proinflammatory cytokines in skin MC than in blood-derived monocytes (Table 1) are not a contradiction to this hypothesis, as the proinflammatory potential of the latter is known to decrease during maturation toward tissue macrophages [41], and this also applies clearly to macrophages in human skin (own unpublished results).

Like mucosal MC [10], skin MC acquire the ability to produce selected Th2 cytokines (in particular, IL-5) after long-term priming by IL-4. Although the repertoire of these cytokines seems more limited in skin MC than in intestinal MC [10, 11] (as IL-3 and IL-13 are not induced by IL-4 pretreatment in the former), this is an important finding corroborating the marked plasticity of human MC, even after their maturation within tissues has been completed. Therefore, alterations to the cells' microenvironment, which may be found in allergic disorders, could alter the range of MC cytokines, with de novo appearance of IL-5 (and concomitant down-regulation of IL-1 β and IL-6). Together with other interplayers, IL-5 would strongly favor the recruitment and activation of eosinophils rather than neutrophils (as under baseline conditions) [42], with eosinophil-derived mediators (including IL-4) inducing positive-feedback regulation and circuits of amplification, thereby helping sustain allergic inflammation.

The selective alteration in a differentiated cell induced by an external stimulus (such as IL-4) is again not a purely MC-specific phenomenon but has been well documented in the past for macrophages, where classically activated, proinflam-

matory, and pathogen-killing cells (M Φ 1) can be distinguished from alternatively activated, regulatory cells (M Φ 2), the latter of which favor cell growth, fibrosis, and angiogenesis [43, 44]. Our data and those from the recent literature [10, 11] underline that an analogous polarization also occurs in MC, although the mediators induced by IL-4 in the two myelocytes may not be completely alike [43, 44]. The switching capacity as such, however, is of interest and shows a significant adaptability and flexibility of both myeloid tissue cells, also further substantiating a close relation between the two lineages [39, 45].

Together, our data have shown that human skin MC are constitutively equipped with the capacity to mount a response against intruding pathogens by producing mainly proinflammatory cytokines. This function may be most vital. However, MC (like macrophages) retain the ability to adjust to changes in their microenvironment, such as the de novo appearance of IL-4, resulting in their polarization and significant shifts in the repertoire of mediators.

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