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Ontogenesis of protein kinase C βII and its anchoring protein RACK1 in the maturation of alveolar macrophage functional responses

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Abstract

Alveolar macrophages are the resident airway cells primarily responsible for the protection of the lungs against inhaled toxins and other biologically active materials. The purpose of this study was to investigate the maturation with age of alveolar macrophage functional responses. We characterised the ontogenesis of PKC βII and its anchoring protein RACK1 in correlation with PKC-dependent immune functions, such as TNF-α, hydrogen peroxide production and lysozyme release in resident alveolar macrophages obtained from rats 2, 4 and 12 weeks old. Our results show an age-associated increase in the expression of PKC βII and RACK1, which correlated with a maturation of alveolar macrophage functional responses.

Keywords: Ontogenesis; Natural immunity; Lysozyme; Tumor necrosis factor-α; Hydrogen peroxide; RACK1

1. Introduction

The lung presents the largest epithelial surface area of the body to the external environment. Airways are repeatedly exposed to a multitude of particles and microorganisms. An elaborate defence system is in place to maintain the lung's sterility [1].

Very young and old people are considered at risk for infections. In infants, the immune system is developing, while in the elderly the immune functions are declining. Respiratory infections, especially pneumonia, are common in the first 4 weeks of life and are the cause of significant morbidity and mortality [2–5]. The vulnerability of infants to infective agents depends on interactions between genetic, developmental and environmental factors that contribute to colonisation by microorganisms; the inflammatory and specific immune responses, and the infants' physiological responses to inflammatory mediators [6], whereas in the aged population, immunosenescence is a major predisposing factor to the increased incidence, morbidity, and mortality of pneumonia [7,8].

Alveolar macrophages are the resident airway cells, situated at the air-tissue interface in the alveoli and alveolar ducts, primarily responsible for the protection of the lungs against inhaled toxins and other biologically active materials. These cells can phagocytise and inactivate foreign materials by production of reactive oxygen species and the action of hydrolytic enzymes. In particular, hydroxyl radical and proteases such as lysozyme, are potent bactericidal agents. They can also produce arachidonic acid metabolites and cytokines that contribute to the inflammatory response of the lungs [9].

We have recently demonstrated that the age-associated decline in alveolar macrophage functions reflects an impaired PKC signal transduction pathway, and

Abbreviations: BAL, bronchoalveolar lavage; IFN-γ, interferon-γ; LPS, lipopolysaccharide; PKC, protein kinase C; RACKl, receptor for activated C kinase-l; TNF-α, tumor necrosis factor-α; TPA, 12-*O*-tetradecanoylphorbol-l3-acetate.

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correlates with a defective PKC anchoring system [10]. PKC is a family of phospholipid-dependent serine-threonine kinases involved in the signal transduction of hormones, neurotransmitters, and cytokines [11]. Molecular cloning and biochemical studies have provided a basis for classifying the different PKC isoenzymes into calcium-dependent (α, βI, βII and γ) and calcium independent (novel PKC -δ, -ε, -η, θ, and μ, atypical PKC -ζ, ι, and λ) species. The activation of PKC results in redistribution (translocation) of the enzyme from the cytosol to membrane compartments [12]. Recently, a family of proteins that interacts with PKC has been described (for a recent review see [13]). These receptors for activated C kinase (RACKs) are 30–36 kDa proteins located in various subcellular compartments. RACK1, a 36-kDa protein cloned from rat brain, is the best-characterised member of the RACK family. It has been shown that RACK1 preferentially interacts with PKC β as compared with other PKC isoforms. We have shown that a deficit in RACK1, even when there were no differences in the expression of LPS receptor or total PKC, contributes to the functional impairment in aged alveolar macrophages [10].

The purpose of this study was to investigate, if a similar situation could be found in very young animals, contributing to explain the increased susceptibility to pulmonary infections in infants. In particular, we characterised in resident alveolar macrophages obtained from rats at 2, 4 and 12 weeks of age, the ontogenesis of PKC βII and its anchoring protein. Our results showed an age-associated increase in the expression of PKC βII and RACK1, which correlated with a maturation of alveolar macrophage functional responses, namely increase in TNF-α, hydrogen peroxide production and lysozyme release.

2. Materials and methods

2.1. Animals

The experiments were performed with alveolar macrophages obtained from male Sprague–Dawley rats of different ages (Charles River, Calco, Italy). All animal care procedures were in accordance with the local Animal Care Committee, and no deaths were observed after receipt of rats in our animal facility. Rats were acclimatised to a 12-h light:12-h dark cycle.

2.2. Chemicals

LPS from *Escherichia coli* serotype 0127:B12 was obtained from Sigma (St. Louis, MO). Recombinant murine TNF-α (specific activity, 4×10^7 U/mg) was obtained from Genzyme (Cambridge, MA), antibodies against RACK1 and murine CD14 were obtained from Transduction Laboratories (Affinity, Nottingham, UK) and Santa Cruz Biotechnology (Santa Cruz, CA), respectively. The antisera raised against PKC βII has been earlier characterised [14]. Salts were purchased from Sigma, and electrophoresis reagents were from BioRad (Richmond, CA). All reagents were purchased at the highest purity available.

2.3. Cells

Alveolar macrophages were collected by lavaging the lungs as earlier described [15]. Recovery ranged from $2–15 \times 10^6$ cells/animal depending on its age, $>98\%$ being macrophages (Giemsa staining). Once washed and resuspended to 10^6 viable alveolar macrophages per ml for functional assays, cells were allowed to adhere to plastic plates in serum-free RPMI 1640 (Sigma) containing 2 mM L-glutamine, 0.1 mg/ml streptomycin, 100 IU/ml penicillin, and 50 ng/ml gentamicin (medium). After adhesion for 1 h at 37°C in 5% CO_2, the plates were washed once with warm medium to remove nonadherent cells. Cells were then exposed to medium with 10% FCS (Sigma) and incubated with or without LPS (10 and 100 ng/ml) for 24 h for the determination of TNF-α and lysozyme release (see below). For hydrogen peroxide production, cells were primed for 48 h with interferon-γ 1000 U/ml, and then TPA 50 ng/ml or rat serum opsonised zymosan 200 μg/ml was added for 2 h (see below).

2.4. Assay for TNF-α

TNF-α concentration in culture supernates was assayed by determining the cytotoxicity of TNF-α against sensitive L929 cells [16] and calculated against a standard curve based on recombinant murine TNF-α.

2.5. Assay for lysozyme

Lysozyme levels were determined in the conditioned medium by a spectrophotometric assay [17]. Alveolar macrophages in medium with 10% fetal calf serum (FCS) were cultured for 24 h and then 100 μl of the supernate was assayed for lysozyme. Lysozyme from turkey egg white (Sigma, 82 000 U/mg protein) was used as a standard.

2.6. Assay for hydrogen peroxide

Hydrogen peroxide was measured [18] as nmol H_2O_2 per 10^5 cells per 2 h.

2.7. Western blot analysis

For CD14, PKC βII, and RACK1, 5×10^6 alveolar macrophages obtained from rats of different ages were

lysed in 100 μl of homogenisation buffer (50 mM Tris, 150 mM NaCl, 5 mM EDTA (pH 7.5), 0.5% Triton X-100, 50 μM PMSF, 2 μg/ml aprotinin, 1 μg/ml pepstatin, and 1 μg/ml leupeptin) and denatured in 100 μl Laemmli sample buffer [19] for 5 min at 100°C. The protein content of the cell lysate was measured using a commercial kit (Bio-Rad). About 10 μg of protein were electrophoresed into a 12% SDS-polyacrylamide gel under reducing conditions and then transferred to nitrocellulose membrane. The different proteins were visualised with a CD14 antiserum diluted at 1:200, RACK1 at 1:2500, and PKC βII at 1:5000, as primary antibodies and developed using enhanced chemoluminescence (ECL, Amersham, Buckinghamshire, UK).

3. Results

Alveolar macrophages recovered by bronchoalveolar lavage from male Sprague–Dawley rats at 2, 4, and 12 weeks (> 98% were macrophages) increased in number with rat age. After cytosine and Giemsa staining, the cells recovered (BAL cells) were morphologically similar in the three ages, with a slight increase in cell size between 2 and 4 weeks of age (Fig. 1).

Isolated alveolar macrophages were analysed for functional responses and for PCK βII, RACK1 or CD14 expression by Western blot analysis. Macrophage functions considered were TNF-α release in response to LPS, hydrogen peroxide production after IFN-γ priming and TPA (direct activator of PKC) or opsonised zymosan (receptor-dependent stimulus) stimulation, and, finally, lysozyme release. Fig. 2 shows a progressive functional maturation with age for all functional parameters considered. All alveolar macrophages responded in a dose-dependent manner to LPS stimulation (Fig. 2A), but the absolute amount of TNF-α released, increased markedly with age. A less dramatic but still significant increase in hydrogen peroxide and lysozyme production occurs as a function of age (Fig. 2B and C). Fig. 3 demonstrates that in parallel with the maturation of macrophages functions, there is a similar progressive increase with age in the expression of PKC βII and its anchoring protein RACK1.

4. Discussion

We have demonstrated that rat alveolar macrophages increase their main immunoprotective functions during development. As shown by the data, all of the parameters considered, increase with age within the interval between 1 and 12 weeks.

The most dramatic changes were observed in the release of TNF-α in response to LPS stimulation. TNF-α is rapidly produced following, either antigen-specific or non-specific stimulation and is considered an early-response cytokine. TNF-α produced by alveolar macrophages serve as mediator, through C-X-C chemokine induction, in eliciting the recruitment of PMNs into the lung [20]. Furthermore, neutralisation of TNF-α has been shown to impair pulmonary host defence against a wide range of pathogens [21,22]. Differences in LPS receptor expression (CDl4) could not account for the differences in TNF-α release, since (Fig. 3) similar amounts of CD14 were expressed at the three ages.

A similar functional maturation was also observed for lysozyme release (Fig. 2C). In several experimental models of monocyte-macrophage differentiation, the development of fully differentiated macrophages is associated with elevated lysozyme activity [23,24], and the expression of lysozyme gene is a standard for indicating

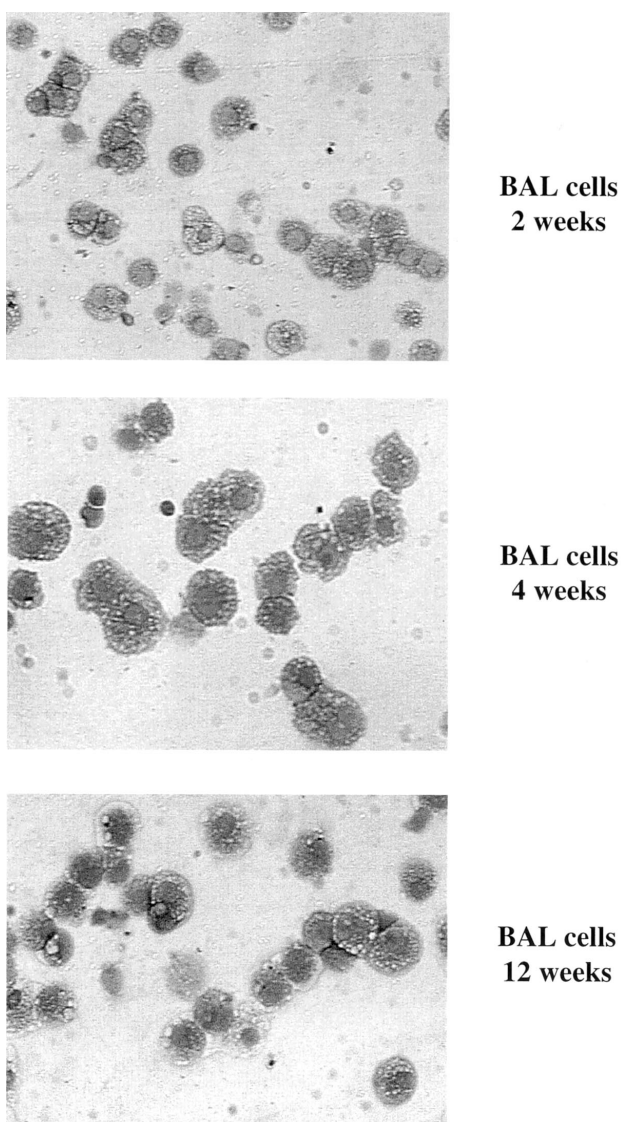

Fig. 1. BAL cells recovered from 2, 4, and 12-week-old rats. After isolation, cells were stained by Giemsa 20X magnification.

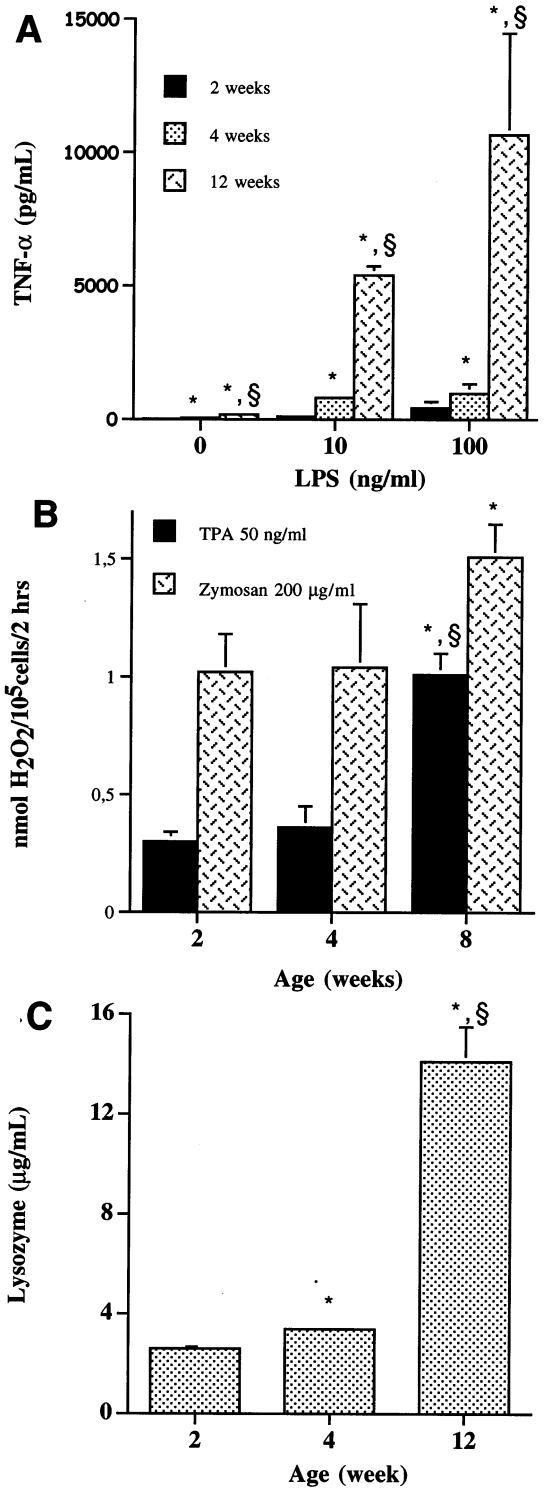

Fig. 2. Ontogenesis of alveolar macrophage functional responses. Panel A, LPS-induced TNF-α release at 2, 4, and 12 weeks. Cells were treated for 24 h with 0, 10 and 100 ng/ml LPS. Panel B, hydrogen peroxide production following TPA (40 ng/ml) or rat serum opsonised zymosan for 2 h. Panel C, release of lysozyme after 24 h of incubation. Mean ± standard deviation (S.D.) of three to four determinations. All experiments were performed three times, with representative results shown. Statistical evaluation was performed with Dunnett's muliple comparison test, with $*p < 0.01$ vs 2 weeks old animals and $§p < 0.01$ vs 4 weeks old animals.

the proportion of macrophage cells in the differentiating culture [25].

All the parameters considered, including hydrogen peroxide production (see below) have been reported in the literature to be dependent on PKC activation, and all are recognised as key mediators of the host response to infections.

We were able to demonstrate that the maturation of alveolar macrophage functional responses correlates with an age-associated increase in the expression of PKC βII and its anchoring protein RACK1. We focussed our attention on the expression of PKC βII and its anchoring protein RACK1, because, we have earlier demonstrated that the age-associated decline in alveolar macrophage functions correlates with a defective PKC βII anchoring system, since the use of RACK1 antisense oligonucleotides reduces the ability of alveolar macrophages to respond to LPS [10]. These observations suggest that the same system is a likely candidate to explain also developmental changes. The differences described in all functional responses are therefore, related to the maturation of the PKC system.

The immature PKC system in young animals could also account for the limited hydrogen peroxide production, especially following TPA stimulation as shown in Fig. 2B. Also, in this case, an age-associated increase in hydrogen peroxide was observed. This effect was less evident following rat serum opsonised zymosan. This is in agreement with earlier findings [26], showing that hydrogen peroxide production in response to zymosan particles was only slightly attenuated in macrophages, depleted of PKC by prolonged exposure to TPA.

By the age of 12 weeks, young adult rats possess fully matured alveolar macrophages with respect to their ability to respond to stimuli as well as a fully mature PKC βII/RACK1 signal transduction system. At present, however, it is not clear what events upstream of the expression of functional signal transduction systems are responsible for the development of macrophages immunocompetence. Lung macrophages

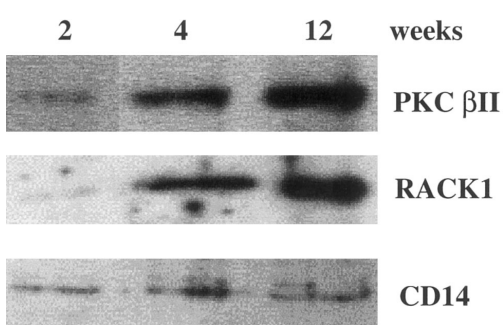

Fig. 3. Ontogenesis of PKC βII, RACK1 and CDl4 expression in alveolar macrophages obtained from 2, 4, and 12 week old rats. Representative Western blot analysis of PKC βII, RACK1, and CD14 immunoreactivity in cell homogenates from resident alveolar macrophages. Protein was loaded at 10 µg.

arise from circulating blood monocytes and undergo further maturation during the migration into the lung tissue. Both exogenous and endogenous regulatory factors participate to this maturation process [27].

Our findings contribute to the understanding of alveolar macrophage maturation and may explain the mechanism of the higher susceptibility to pulmonary infections in infants.

References

[1] P. Zhang, W.R. Summer, G.J. Bagdy, S. Nelson, Immunol. Rev. 173 (2000) 39–51.
[2] P.H. Dennehy, Clin. Perinatol. 14 (1987) 667–682.
[3] J.R. Campbell, Sem. Respir. Infect. 11 (1996) 155–162.
[4] W.W. Busse, Rev. Infect. Dis. 13 (1991) S477–485.
[5] C.H. Wendt, M.I. Hertz, Sem. Respir. Infect. 10 (1995) 224–231.
[6] C.C. Blackwell, D.M. Weir, A. Busuttil, A.T. Saadi, S.D. Essery, M.W. Raza, V.S James, D.A. Mackenzie, FEMS Immunol. Med. Microbiol. 9 (1994) 91–100.
[7] R.J. Simons, H.Y. Reynolds, Sem. Respir. Infect. 5 (1990) 251–259.
[8] S. Antonacci, E. Jirillo, A. Ventura, R. Garofano, L. Bonomo, Mech. Ageing Dev. 24 (1984) 367–375.
[9] Y. Sibille, H.Y. Reynolds, Am. Rev. Respir. Dis. 141 (1990) 471–501.
[10] E. Corsini, F. Battaini, L. Lucchi, M. Marinovich, M. Racchi, S. Govoni, C.L. Galli, J. Immunol. 163 (1999) 3468–3473.
[11] L.V. Dekker, P.J. Parker, Trends Biochem. Sci. 19 (1994) 73–77.
[12] Y. Nishizuka, FASEB J. 9 (1995) 484–496.
[13] D. Mochly-Rosen, H. Khaner, J. Lopez, Proc. Natl. Acad. Sci. USA 88 (1991) 3997–4000.
[14] W.C. Wetsel, W.A. Khan, I. Merchenthaler, H. Rivera, A.E. Halpern, H.M. Phung, A. NegroVilar, Y.A. Hannun, J. Cell Biol. 117 (1992) 212–233.
[15] E. Corsini, W.A. Craig, G.J. Rosenthal, Int. J. Immunopharmacol. 14 (1992) 121–130.
[16] G.J. Rosenthal, E. Corsini, in: G.R. Burleson, J.H. Dean, A.E. Munson (Eds.), Methods in Immunotoxicology, vol. 2, Wiley, New York, 1995, pp. 327–343.
[17] L.A. Nerurkar, in: D.O. Adams, P.J. Edelson, H. Koren (Eds.), Methods for Studying Mononuclear Phagocytes, Academic Press, New York, 1981, pp. 667–683.
[18] K. Rodgers, in: G.R. Burleson, J.H. Dean, A.E. Munson (Eds.), Methods in Immunotoxicology, vol. 2, Wiley, New York, 1995, pp. 67–77.
[19] V.K. Laemmli, Nature 227 (1970) 680–685.
[20] S.L. Kunkel, T. Standford, K. Kassahara, R.M. Strieter, Exp. Lung Res. 17 (1991) 17–23.
[21] S. Nelson, G.J Bagby, W.R. Summer, Am. Rev. Respir.Dis. 143 (1991) S393.
[22] L.L. Laichalk, S.L. Kunkel, R.M. Strieter, Infect. Immun. 64 (1996) 5211–5218.
[23] M. Kreutz, R. Andreesenm, Blood 76 (1990) 2457–2461.
[24] Y. Uemura, M. Nishikawa, F. Komada, S. Shirikawa, Leuk. Res. 13 (1989) 545–552.
[25] N. Faust, C. Bonifer, M.V. Wiles, A.E. Sippel, DNA Cell Biol. 13 (1994) 901–907.
[26] P.H. Sporn, T.M. Marshall, M. Peters-Golden, Biochim. Biophys. Acta 1047 (1990) 187–191.
[27] D.J. Laszlo, Am. J. Pathol. 143 (1992) 587–597.