sion of BAFF molecule. However, data in the experiments to clarify whether the effect of CAM on upregulation of BAFF on DCs is a direct or indirect effect suggest that CAM indirectly enhances BAFF expression on mucosal DCs (Table 1). Further screening studies for the target molecule(s) of CAM are currently in progress.

In summary, using IAV-infected weanling mice, the present study demonstrated that orally administered CAM enhances mucosal IAV-specific S-IgA immune responses and has neutralization activities. Thus, based on the production of mucosal S-IgA Ab, we consider that the combination of CAM-OSV treatment for IAV could potentially prevent complications and aggravation of the flu symptoms at an early stage of infection.

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The authors declare they have no potential conflicts of interest.

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REFERENCES

1. Al-Garawi AA, et al. 2009. Acute, but not resolved, influenza A infection enhances susceptibility to house dust mite-induced allergic disease. J. Immunol. 182:3095–3104.
2. Barone F, Patel P, Sanderson JD, Spencer J. 2009. Gut-associated lymphoid tissue contains the molecular machinery to support T-cell-dependent and T-cell-independent class switch recombination. Mucosal Immunol. 2:495–503.
3. Brandtzaeg P, Farstad IN, Haraldsen G. 1999. Regional specialization in the mucosal immune system: primed cells do not always home along the same track. Immunol. Today 20:267–277.
4. Castigli E, et al. 2004. Impaired IgA class switching in APRIL-deficient mice. Proc. Natl. Acad. Sci. U. S. A. 101:3903–3908.
5. Castigli E, et al. 2005. TACI and BAFF-R mediate isotype switching in B cells. J. Exp. Med. 201:35–39.
6. Cerutti A. 2008. The regulation of IgA class switching. Nat. Rev. Immunol. 8:421–434.
7. Choi YS, Baumgarth N. 2008. Dual role for B-1a cells in immunity to influenza virus infection. J. Exp. Med. 205:3053–3064.
8. Crouch EE, et al. 2007. Regulation of AID expression in the immune response. J. Exp. Med. 04:1145–1156.
9. Fagarasan S, Kinoshita K, Muramatsu M, Ikuta K, Honjo T. 2001. *In situ* class switching and differentiation to IgA-producing cells in the gut lamina propria. Nature 413:639–643.
10. Hamada H, et al. 2002. Identification of multiple isolated lymphoid follicles on the antimesenteric wall of the mouse small intestine. J. Immunol. 168:57–64.
11. Hashisaki GT. 1995. Update on macrolide antibiotics. Am. J. Otolaryngol. 16:153–157.
12. He B, et al. 2007. Intestinal bacteria trigger T cell-independent immunoglobulin A2 class switching by inducing epithelial cell secretion of the cytokine APRIL. Immunity 26:812–826.
13. Honeybourne D, Kees F, Andrews JM, Baldwin D, Wise R. 1994. The levels of clarithromycin and its 14-hydroxy metabolite in the lung. Eur. Respir. J. 7:1275–1280.
14. Kataoka K, et al. 2011. Nasal dendritic cell targeting flt3 ligand as a safe adjuvant elicits effective protection against fatal pneumococcal pneumoniae. Infect. Immun. 79:2819–2828.
15. Kataoka K, et al. 2007. Nasal cholera toxin elicits IL-5 and IL-5 receptor alpha-chain expressing B-1a B cells for innate mucosal antibody responses. J. Immunol. 178:6058–6065.
16. Kataoka K, et al. 2011. Oral-nasopharyngeal dendritic cells mediate T cell-independent IgA class switching on B-1 B cells. PLoS One 6:e25396. doi:10.1371/journal.pone.0025396.
17. Kataoka K, et al. 2004. Nasal Flt3 ligand cDNA elicits CD11c+CD8+ dendritic cells for enhanced mucosal immunity. J. Immunol. 172:3612–3619.
18. Kido H, Okumura Y, Yamada H, Le TQ, Yano M. 2007. Protease essential for human influenza virus entry into cells and their inhibitors as potential therapeutic agents. Curr. Pharm. Des. 13:405–414.
19. Kido H, et al. 2011. Attenuation of respiratory immune responses by antiviral neuraminidase inhibitor treatment and boost of mucosal immunoglobulin A response by co-administration of immuno-modulator clarithromycin in pediatric influenza. Influenza Other Respir. Viruses 5:240–243.
20. Kinoshita K, Harigai M, Fagarasan S, Muramatsu M, Honjyo T. 2001. A hallmark of active class switch recombination: transcripts directed by I promoters on looped-out circular DNAs. Proc. Natl. Acad. Sci. U. S. A. 98:12620–12633.
21. Kiyono H, Kunisawa J, McGhee JR, Mestecky J. 2008. The mucosal immune system, p 983–1030. *In* Paul WE (ed), Fundamental immunology, 5th ed. Lippincott Williams & Wilkins, Philadelphia, PA.
22. Kunkel EJ, Butcher EC. 2003. Plasma-cell homing. Nat. Rev. Immunol. 3:822–829.
23. Liew FY, Russel SM, Appleyard G, Brand CM, Beale J. 1984. Cross-protection in mice infected with influenza A virus by the respiratory route is correlated with local IgA antibody rather than serum antibody or cytotoxic T cell reactivity. Eur. J. Immunol. 14:350–356.
24. Litinskiy MB, et al. 2002. DCs induce CD40-independent immunoglobulin class switching through BLyS and APRIL. Nat. Immunol. 3:822–829.
25. Maeda S, Yamada Y, Nakamura H, Maeda T. 1999. Efficacy of antibiotics against influenza-like illness in an influenza epidemic. Pediatr. Int. 41:274–276.
26. Miyamoto D, et al. 2008. Clarithromicin inhibits progeny virus production from human influenza virus-infected host cells. Biol. Pharm. Bull. 31:217–222.
27. Mizuno D, Ide-Kurihara M, Ichinomiya T, Kubo I, Kido H. 2006. Modified pulmonary surfactant is a potent adjuvant that stimulates the mucosal IgA production in response to the influenza virus antigen. J. Immunol. 176:1122–1130.
28. Mizuno D, et al. 2011. Surfactant protein C is an essential constituent for mucosal adjuvanticity of Surfacten, acting as an antigen delivery vehicle and inducing both local and systemic immunity. Vaccine 29:5368–5378.
29. Mora JR, et al. 2006. Generation of gut-homing IgA-secreting B cells by intestinal dendritic cells. Science 314:1157–1160.
30. Muramatsu M, et al. 1999. Specific expression of activation-induced cytidine deaminase (AID), a novel member of the RNS-editing deaminase family in germinal center B cells. J. Biol. Chem. 274:18470–18476.
31. Nguyen HH, et al. 1999. Heterosubtypic immunity to lethal influenza A virus infection is associated with virus-specific CD8+ cytotoxic T lymphocyte responses induced in mucosa-associated tissues. Virology 254:50–60.
32. Nguyen HH, van Ginkel FW, Vu HL, McGhee JR, Mestecky J. 2001. Heterosubtypic immunity to influenza A virus infection requires B cells but not CD8+ cytotoxic T lymphocytes. J. Infect. Dis. 183:368–376.
33. Nishino M, et al. 2009. Influenza vaccine with Surfacten, a modified pulmonary surfactant, induces systemic and mucosal immune responses without side effects in minipigs. Vaccine 27:5620–5627.
34. Okumura Y, et al. 2006. Serase-1B, a new splice variant of polyserase-1/TMPRSS9, activates urokinase-type plasminogen activator and proteolytic activation is negatively regulated by glycosaminoglycans. Biochem. J. 400:551–561.
35. Pan H-Y, et al. 2011. Up-regulation of ectopic trypsins in the myocardium by influenza A virus infection triggers acute myocarditis. Cardiovasc. Res. 89:595–603.
36. Sato K, et al. 1998. Therapeutic effect of erythromycin of influenza virus-induced lung injury in mice. Am. J. Respir. Crit. Care Med. 157:853–857.
37. Sawabuchi T, et al. 2009. Boost of mucosal secretory immunoglobulin A response by clarithromycin in pediatric influenza. Respirology 14:1173–1179.
38. Shikina T, et al. 2004. IgA class switch occurs in the organized nasopharynx- and gut-associated lymphoid tissue, but not in the diffuse lamina propria of airways and gut. J. Immunol. 172:6259–6264.
39. Takahashi E, et al. 2010. Attenuation of inducible respiratory immune

responses by oseltamivir treatment in mice infected with influenza A virus. Microbes Infect. 12:778–783.
40. Tsuji M, et al. 2008. Requirement for lymphoid tissue-inducer cells in isolated follicle formation and T cell-independent immunoglobulin A generation in the gut. Immunity 29:261–271.
41. Tumpey TM, Renshaw M, Clements JD, Katz JM. 2001. Mucosal delivery of inactivated influenza vaccine induces B-cell-dependent heterosubtypic cross-protection against lethal influenza A H5N1 virus infection. J. Virol. 75:5141–5150.
42. Uematsu S, et al. 2008. Regulation of humoral and cellular gut immunity by lamina propria dendritic cells expressing Toll-like receptor 5. Nat. Immunol. 9:769–776.
43. Yamada T, et al. 2010. Poly(I:C) induces BLyS-expression of airway fibroblasts through phosphatidylinositol 3-kinase. Cytokine 50:163–169.
44. Yamada T, Zhang K, Yamada A, Zhu D, Saxon A. 2005. B lymphocyte stimulator activates p38 mitogen-activated protein kinase in human Ig class switch recombination. Am. J. Respir. Cell Mol. Biol. 32:388–394.
45. Yamaya M, et al. 2010. Clarithromycin inhibits type A seasonal influenza virus infection in human airway epithelial cells. J. Pharmacol. Exp. Ther. 333:81–90.

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Original Article

Intranasal influenza vaccination using a new synthetic mucosal adjuvant SF-10: induction of potent local and systemic immunity with balanced Th1 and Th2 responses

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Background We found previously that bovine pulmonary Surfacten® used in newborns with acute respiratory distress syndrome is a safe and efficacious antigen vehicle for intranasal vaccination.

Objectives The objective of this study was to industrially produce a synthetic adjuvant mimicking Surfacten® for clinical use without risk of bovine spongiform encephalopathy.

Methods We selected three Surfacten lipids and surfactant protein (SP)-C as essential constituents for adjuvanticity. For replacement of the hydrophobic SP-C, we synthesized SP-related peptides and analyzed their adjuvanticity. We evaluated lyophilization to replace sonication for the binding of influenza virus hemagglutinin (HA) to the synthetic adjuvant. We also added a carboxy vinyl polymer (CVP) to the synthetic adjuvant and named the mixture as SF-10 adjuvant. HA combined with SF-10 was administered intranasally to mice, and induction of nasal-wash HA-specific secretory IgA (s-IgA) and serum IgG with Th1-/Th2-type cytokine responses in nasal cavity and virus challenge test were assessed.

Results and Conclusions Intranasal immunization with HA–SF–10 induced significantly higher levels of anti-HA-specific nasal-wash s-IgA and serum IgG than those induced by HA-poly(I:C), a reported potent mucosal vaccine, and provided highly efficient protection against lethal doses of virus challenge in mice. Anti-HA-specific serum IgG levels induced by HA–SF–10 were almost equivalent to those induced by subcutaneous immunization of HA twice. Intranasal administration of HA–SF–10 induced balanced anti-HA-specific IgG1 and IgG2a in sera and IFN-γ- and IL-4-producing lymphocytes in nasal cavity without any induction of anti-HA IgE. The results suggest that HA–SF–10 is a promising nasal influenza vaccine and that SF-10 can be supplied in large quantities commercially.

Keywords influenza vaccine, nasal vaccination, Pulmonary surfactant, synthetic mucosal adjuvant, Th1/Th2 responses.

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Introduction

The recent global spread of swine-origin H1N1 influenza A virus (IAV) highlighted the need for the development of effective vaccines for the prevention of viral infection and transmission. The currently available influenza vaccines administered intramuscularly or subcutaneously induce a predominantly IgG-mediated protection in the systemic immune compartment and significantly reduce hospitalization and deaths when they match antigenically the circulating viral strains.[1,2] However, this immunization results in neither adequate induction of antiviral secretory IgA (s-IgA), which provides a wide cross-protection, nor efficient prevention of the initial airway infection[3,4] or cell-mediated responses in the upper and lower respiratory tracts.[5] To address the need for improved influenza vaccines, nasal vaccination that stimulates both mucosal and systemic immunity[4] is desirable for future vaccination against airway viral infection.

Mucosal vaccines and adjuvants have been studied for over 40 years[6,7]; however, many were found to be ineffective or have safety problems.[8] There are two key issues with regard to nasal vaccines.[9] One is the poor efficiency of antigen uptake across the nasal mucosa due to mucociliary clearance. The other is the safety issue; that is, protection against hyperstimulation of antigen-presenting cells or unexpected antibody induction. At present, the cold-adapted live flu mucosal vaccine, FluMist®, is available in the USA[10] for individuals aged 2–49 years and has been licensed in Europe from 2012 under the name FLUENZ for children aged 2–17 years. It is not licensed for children <2 years of age where

it is known to potentially cause post-vaccination flu symptoms with severe wheezing.[11,12]

To overcome these issues, we recently reported that natural pulmonary surfactant and its commercially available bovine product, Surfacten®, which has been used as a natural replacement medicine in premature babies for more than 25 years without significant adverse effects,[13] show safety and efficacy of mucosal adjuvanticities by promoting antigen delivery to antigen-presenting cells in mice and minipigs.[9,14,15] The lung surfactant is effectively uptaked into alveolar cells, macrophages, and dendritic cells and rapidly metabolized *in vivo* with a short half-life of 6–7 hour in the lungs.[16] In addition, we recently found that three major Surfacten lipids and surfactant protein C (SP-C) are essential constituents for mucosal adjuvanticity of Surfacten.[9] In mammals, SP-C is a 33- to 35-residue lipopeptide that consists of a hydrophobic transmembrane α-helix and a cationic N-terminal segment and plays an important role in the uptake of surfactant lipids to alveolar macrophages and epithelial cells.[17] To provide ample supply of the mucosal adjuvant for clinical use, it is important to commercially develop a synthetic compound that carries no risk of bovine spongiform encephalopathy.

In this study, we describe an effective preparation process of a synthetic surfactant (SSF) and a further improved synthetic mucosal adjuvant SF-10 mimicking Surfacten for large-scale manufacturing by improving the following issues. As a substitute for SP-C(1–35), which does not dissolve easily in common organic solvents, we identified a methanol-soluble SP-related peptide. The formation of a complex between influenza hemagglutinin vaccine (HA) and SSF was improved for large-scale manufacturing by lyophilization instead of sonication. In addition, we found a mucoadhesive additive to increase the viscosity of HA-SSF mixture to avoid rapid clearance from the nasal cavity and termed the improved adjuvant compound SF-10. Based on these improvements, we analyzed the enhancement of mucosal and systemic immunity by SF-10 and the resultant Th1- and Th2-type cytokine responses and protective immunity in mice.

Materials and methods

Animals and virus

All experiments were performed in 6- to 8-week-old BALB/c female mice obtained from Japan SLC, Inc. (Shizuoka, Japan). All animals were treated according to the Guide for the Care and Use of Laboratory Animals (NIH Publication No. 85-23, 1996), and the study was approved by the Animal Care Committee of the Tokushima University. IAV/ PR8/34 (H1N1) and A/New Caledonia/20/99(H1N1) were provided by The Research Foundation for Microbial Diseases of Osaka University (Kagawa, Japan).

Reagents

Surfacten® was purchased from Mitsubishi Pharma (Tokyo, Japan). 1,2-Dipalmitoyl-phosphatidylcholine (DPPC), phosphatidylglycerol (PG), and palmitate (PA) for the preparation of SSF were obtained from Nippon Fine Chemical (Osaka, Japan). Synthetic SP-related peptides (Table 1; >80% grade) were obtained from Greiner (Frickenhausen, Germany). A carboxy vinyl polymer (CVP) was purchased from Sigma-Aldrich (St. Louis, MO, USA) and poly(I:C) from Alexis Biochemicals (Lausen, Switzerland).

Preparation of antigen and SSF

IAV/New Caledonia/20/99(H1N1) split antigen (0·2 μg of viral protein, corresponding to 0·14 μg HA; Denka Seiken, Tokyo, Japan) was used as a HA antigen in the present studies. SSF was prepared by mixing three lipids, DPPC, PG, and PA, and various SP-related synthetic peptides, at a molar ratio of 75:25:30:0·6, respectively, as described previously.[9] SSF samples (4 mg phospholipids/ml) were then lyophilized for storage.

Immunization and virus challenge

Lyophilized SSF (its weight was expressed as that of phospholipids) was suspended in distilled water and then mixed with HA (its weight was expressed as protein) in saline at a ratio of 10:1 (wt/wt) as described previously.[9] The SSF–HA complex formation was carried out by sonication or lyophilization. Sonication method[9]: A mixture of SSF and

Table 1. Amino acid sequences of peptides derived from SP-B and SP-C

	Amino acid sequence
SP-B-type peptide	
SP-B (1–25)	FPIPLPYCWLCRALIKRIQAMIPKG
SP-B (20–60)	AMIPKGALAVAVAQVCRVVPLVAGGICQCLAERYSVILLDT
SP-B (64–80)	RMLPQLVCRLVLRCSMD
KL4	KLLLLKLLLLKLLLLKLLLLK
SP-C-type peptide	
SP-C (1–35)	FGIPCCPVHLKRLLIVVVVVVLIVVVIVGALLMGL
SP-C (1–12)	FGIPCCPVHLKR
SP-C (1–19)	FGIPCCPVHLKRLLIVVVV
SP-C (13–35)	LLIVVVVVVLIVVVIVGALLMGL
SP-CL11	PVHLKRLLLLLLLLLLL
SP-CL16	PVHLKRLLLLLLLLLLLLLLL
K6L16	KKKKKKLLLLLLLLLLLLLLL

HA was treated for 3 minute in a sonic oscillator (model S-250D; Branson Ultrasonics, Danbury, CT, USA) followed by upside-down mixing every 30 minute for 2 hour at room temperature and then stored at 4°C.

Lyophilization method

A mixture of SSF and HA was incubated at 42°C, the critical temperature of Surfacten lipids, for 10 minute with gentle mixing, followed by freezing at −75°C, and then lyophilized. Lyophilized HA + SSF was dissolved in saline before use. Just before administration to mice, CVP in saline at pH 7·0 was added to HA-SSF solution at a final concentration of 0·5%, and the final solution (HA + SSF + CVP) was renamed as HA–SF–10. The amount of HA bound to SSF was calculated by estimating the amount of unbound HA in the SSF-free supernatant fraction after centrifugation as described previously.[9] However, the percentage binding of HA to SF-10 could not be measured due to the inability to separate unbound HA from HA–SF–10 complex by centrifugation or ultrafiltration because of increased viscosity after the addition of CVP.

Mice were immunized by intranasal instillation two or three times every 2 weeks with the prepared samples (2 μl) into each nostril. Positive control mice were subcutaneously injected HA in 100 μl saline under the above immunization schedule. Two weeks after the last immunization, serum and nasal washes were prepared as described previously.[9,14]

For virus challenge experiments, immunized mice were infected with IAV/New Caledonia/20/99(H1N1) at 5×10^4 plaque-forming unit (PFU) by intranasal instillation, for the measurement of neutralization activities of nasal washes. At day 4 after the challenge, virus titers were measured in nasal and lung washes by the plaque assay using Madin-Darby canine kidney cells, which is based on the detection of infected cells using anti-IAV nucleoprotein monoclonal antibody, as described previously.[18]

Immunized mice were also infected with lethal doses of highly pathogenic IAV/ PR8/34(H1N1; 50 and 800 PFU) in 20 μl saline, and the survival rate was monitored for 14 days.

Analysis of mucosal immune responses by ELISA and ELISPOT

Two weeks after the last immunization, serum and nasal-wash specimens were prepared as described previously[14] and subjected to enzyme-linked immunosorbent assay (ELISA) to determine anti-HA-specific s-IgA, IgG, IgG1, IgG2a, and IgE levels.[9,14,15] We used goat anti-mouse IgA, IgG (Sigma, St. Louis, MO, USA), IgG1, IgG2a, or IgE (Bethyl Laboratories, Montgomery, TX, USA) antibodies conjugated with horseradish peroxidase as the secondary antibodies. The levels of total IgE were determined by mouse IgE ELISA Quantitation Set (Bethyl Laboratories). We used 50 ng/ml of purified mouse anti-HA s-IgA, IgG, IgG1, and IgG2a antibodies, which were prepared as described previously,[14] as standards. The hemagglutination inhibition (HI) titers in serum were analyzed after treatment of samples with receptor-destroying enzyme, and the assay was conducted according to the protocol for HI testing established by the World Health Organization, as reported previously.[15]

Mononuclear cells isolated from nasal passages and nasopharynx-associated lymphoid tissue (NALT) by discontinuous Percoll (GE Healthcare, Buckinghamshire, England) density gradient centrifugation[19] were subjected to enzyme-linked immunosorbent spot (ELISPOT) assay, as described previously.[18] The numbers of IL-4- and IFN-γ-producing cells were counted by Mouse ELISPOT Set for IL-4 (BD Pharmingen, Franklin Lakes, NJ, USA) and IFN-γ (MABTECH, Nacka Strand, Sweden) according to the protocols provided by the manufacturers.

Neutralizing activity

To assess the neutralizing activities of the s-IgA in nasal washes, the s-IgA fraction was purified with KAPTIV-AE™ IgA affinity column according to the protocol provided by the manufacturer (Tecnogen, Piacenza, Italy). The concentration of s-IgA was measured by Mouse IgA ELISA Quantitation Set (Bethyl Laboratories). IAV/New Caledonia/20/99(H1N1) at 200 PFU was incubated with 100 μl of the serially diluted s-IgA at 37°C for 1 hour and then the virus titers in the mixtures were measured by the plaque assay, as described previously.[9]

Statistical analysis

All data were expressed as mean ± SD. Differences between groups were examined for statistical significance using the unpaired Student's *t*-test. A P value less than 0·05 denoted statistical significance.

Results

Improvement of synthetic mucosal adjuvant SSF for ample supply

We reported previously that SP-C but not SP-B is an essential constituent of Surfacten for mucosal adjuvanticity.[9] SP-C(1–35) with hydrophobic properties, however, is soluble in 100% trifluoroacetic acid but not in common organic solvents, and its industrial production is scarce. Thus, we designed various peptide fragments of SP-C and SP-B and their modifications (Table 1). SSFs were synthesized by mixing three lipid constituents and each synthetic peptide, and the adjuvanticity was analyzed (Table 2). Adjuvanticity of SSFs containing SP-C-related peptides with 11 to 16 hydrophobic amino acid chain length, but not 7, was almost equivalent to that of Surfacten. Although SSF containing the C-terminal-side hydrophobic domain

Table 2. Effects of mucosal adjuvants, Surfacten, and SSF on the induction of HA-specific antibodies

	Anti-HA antibodies (ng/ml)	
	Nasal washes (s-IgA)	Serum (IgG)
Saline	10·1 ± 4·5	195·5 ± 43·1
HA	9·6 ± 7·4	302·0 ± 125·9
HA-St	196·2 ± 67·7	2907·3 ± 1465·0
HA-SSF		
SP-B type		
SP-B(1–25)	52·0 ± 38·7	622·0 ± 163·9
SP-B(20–60)	37·4 ± 29·4	853·9 ± 344·3
SP-B(64–80)	71·1 ± 37·6	625·9 ± 121·6
KL4	88·3 ± 49·3	778·6 ± 325·9
SP-C type		
SP-C(1–35)	238·1 ± 122·0*	2628·9 ± 942·0*
SP-C(1–12)	23·7 ± 25·5	798·0 ± 688·2
SP-C(1–19)	46·4 ± 40·1	735·1 ± 398·6
SP-C(13–35)	318·1 ± 326·6*	2332·0 ± 1079·7*
SP-CL11	136·6 ± 85·1*	3851·3 ± 2164·1*
SP-CL16	209·8 ± 114·3*	2455·2 ± 1674·3*
K6L16	222·7 ± 145·3*	2104·5 ± 941·7*

Mice were treated with intranasal inoculation of 0·2 μg HA with or without 2 μg of St and various SSF twice at 2-week interval. Two weeks after the last immunization, anti-HA-specific s-IgA in nasal washes and anti-HA-specific IgG in sera were assayed. Data are mean ± SD of 10–15 mice.
*The values are almost equivalent to those of HA-St.

peptide SP-C(13–35) showed relatively high values of anti-HA-specific s-IgA in nasal washes and anti-HA-specific IgG in serum, the levels were not consistent with large SD values. SP-C-related peptides with 11 to 16 hydrophobic amino acid chain length and basic residues in the N-terminal side were soluble in methanol or ethanol, and their adjuvanticity was equivalent to that of Surfacten. Based on these results, we selected a peptide K6L16 among the active peptides, SP-C(1–35), SP-C(13–35), SP-CL11, SP-CL16, and K6L16, as a substitute for SP-C(1–35), which is soluble in methanol and easy for handling, in the following experiments.

To achieve effective antigen delivery, we mixed HA and SSF by sonication to make HA-SSF binding complex. However, avoiding heat damage to HA during sonication is difficult. Accordingly, we used lyophilization as an alternative to sonication and to remove water molecules in the interaction space between HA and SSF for the binding. Lyophilization increased the binding of HA to SSF to ≥92% compared with 65–70% by sonication and significantly increased the induced levels of anti-HA-specific s-IgA in nasal washes at 11·5-fold and IgG in sera at 168·5-fold ($P < 0·01$), compared with those by SSF prepared by sonication (Table 3).

To increase the retention time of HA-SSF in the nasal cavity, we added the mucoadhesive polymer CVP to HA-SSF, to increase its viscosity, at a final concentration of 0·5% just before immunization. The addition of CVP to the lyophilization-prepared HA-SSF further enhanced the induction of anti-HA-specific s-IgA in nasal washes significantly at 4·3-fold ($P < 0·05$) and anti-HA-specific serum IgG at 1·3-fold, the induced levels being the highest among the materials tested. Only limited inductions of anti-HA-specific s-IgA and anti-HA-specific IgG were detected in animals immunized with HA+CPV without SSF, suggesting that SSF is an essential constituent for mucosal adjuvanticity in the mixture. The mixture of HA-SSF and CVP, the best antigen–adjuvant complex, was renamed as HA–SF–10 in the following experiments.

Table 3. Evaluation of various mixing procedures of HA and SSF on induction of HA-specific antibodies

Materials			Preparation			Anti-HA antibodies (μg/ml)	
HA	SSF	CVP	Sonication	Lyophilization	HA binding (%)	Nasal washes (s-IgA)	Serum (IgG)
–	–	–	–	–	-	0·0 ± 0·0	0·1 ± 0·1
+	–	–	+	–	-	0·0 ± 0·0	0·5 ± 0·5
+	+	–	+	–	65-70	0·2 ± 0·2	4·3 ± 0·9
+	+	–	–	+	92-98	2·3 ± 1·4¶	724·5 ± 248·1¶
+	–	+	–	–	-	0·3 ± 0·2	4·9 ± 1·6
+	+	+	+	–	-	3·3 ± 0·9¶	568·7 ± 95·2¶
+	+	+	–	+	-	9·9 ± 5·1¶,*	925·0 ± 633·3¶

Mice were immunized intranasally with HA, with SSF and/or CVP in each combination, three times at 2-week interval. Two weeks after the last immunization, anti-HA-specific s-IgA in nasal washes and IgG in sera were measured by ELISA. Data are mean ± SD of 10–15 mice.
¶$P < 0·01$, compared with HA-SSF mixture prepared by sonication.
*$P < 0·05$, compared with HA-SSF mixture prepared by lyophilization.

Mucosal adjuvanticity of HA–SF–10

For the possible clinical use of SF-10, we evaluated the adjuvanticity of HA–SF–10 and its protective immunity compared with subcutaneously administered HA and intranasally administered HA-poly(I:C), which is one of the most potent mucosal vaccines reported,[20] as positive controls (Figure 1). HA administered subcutaneously twice-effectively induced anti-HA-specific IgG in sera, but not s-IgA in nasal washes. In contrast, intranasally administered HA-SF-10 significantly induced both anti-HA-specific s-IgA in nasal washes and anti-HA-specific IgG in sera, when immunization was repeated three times, compared with the levels induced by intranasal administration of HA or saline three times. No further increase in the induction was observed by administration of HA–SF–10 four times (data not shown). In addition, the levels of anti-HA-specific s-IgA in nasal washes and anti-HA-specific IgG in sera induced by intranasally administered HA–SF–10 were significantly higher at 3·7- and 3·9-fold, respectively, than those induced by intranasally administered HA-poly(I:C) three times ($P < 0·05$). Anti-HA-specific IgG levels in sera induced by intranasal administration of HA–SF–10 three times were almost equivalent to those induced by twice subcutaneous administration of HA. The mean HI titers in serum induced by intranasal administration of HA–SF–10 three times were >300 and were in the similar ranges to those induced by subcutaneous administration of HA twice (Figure S1).

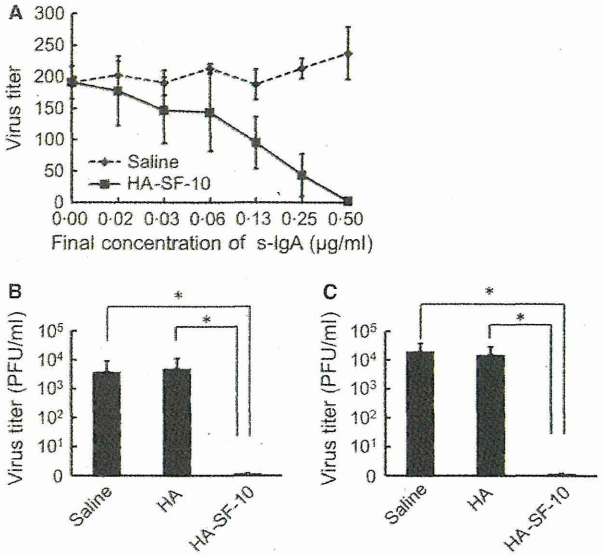

Figure 2. Neutralization activity and protective immunity of nasal washes in mice immunized with intranasal HA–SF–10. (A) Neutralization of IAV/New Caledonia/20/99(H1N1) by isolated s-IgA fraction from nasal washes of mice immunized with intranasal HA–SF–10 or saline. Data are mean virus titer ± SD of three different experiments. (B and C) Two weeks after the last immunization, mice were infected with 5×10^4 PFU of IAV/New Caledonia/20/99(H1N1), and virus titers in nasal washes (B) and lung washes (C) were measured at day 4 after infection. Data are mean ± SD of 8–10 mice. *$P < 0·05$.

Neutralization activities of nasal washes and protective immunity induced by HA–SF–10

In the next step, we assessed the neutralization activities of nasal washes. The neutralizing activity of nasal-wash s-IgA fraction after intranasal immunization with HA–SF–10 is shown in Figure 2A. Nasal-wash s-IgA of mice immunized intranasally with HA–SF–10 neutralized the inoculated IAV/New Caledonia/20/99(H1N1) in a dose-dependent manner, but the fraction obtained from mice treated with saline did not show such activity.

We confirmed the protective immunity in virus challenge experiments (Figure 2B,C). The virus titers in nasal and lung

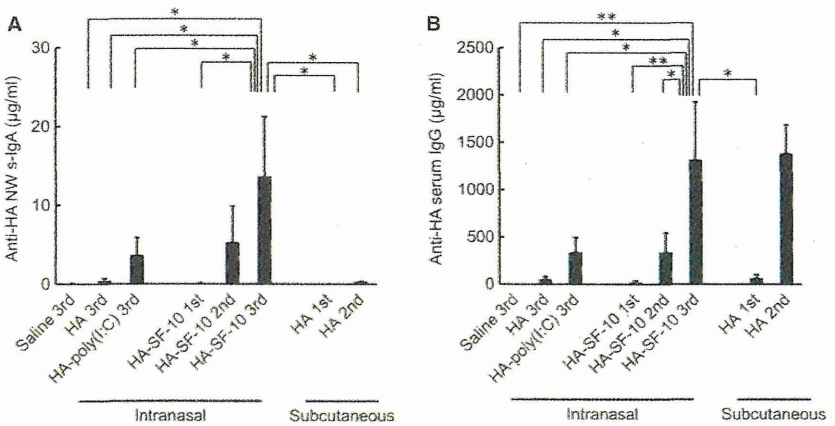

Figure 1. Comparison of the effects of intranasal immunization with HA–SF–10 on mucosal and systemic immunity with those of poly(I:C) and subcutaneous administration of HA. Mice received intranasal HA (0·2 μg) combined with or without SF-10 (2 μg), poly(I:C) (2 μg), or saline, 2 or 3 times every 2 weeks. Another group of mice received subcutaneous HA once or twice every two weeks. After boost inoculation at 2 weeks, the levels of anti-HA-specific s-IgA in nasal washes (NW) (A) and anti-HA-specific IgG in sera (B) were measured. Data are mean ± SD of 5–10 mice. *$P < 0·05$, **$P < 0·01$.

washes were measured after 4-day infection of immunized mice with 5×10^4 PFU of IAV/New Caledonia/20/99 (H1N1). High virus titers were detected in mice immunized with saline or intranasal HA. In contrast, the virus titer was below the detection limit in both nasal and lung washes of mice immunized intranasally with HA–SF–10. We also assessed the protective immunity in mice challenged with two lethal doses ($10 \times LD_{50}$, 50 PFU and $160 \times LD_{50}$, 800 PFU) of IAV/PR8/34(H1N1), a similar subtype but highly pathogenic strain of IAV (Figure 3). All mice that were immunized intranasally with HA–SF–10 survived against the infection with $10 \times LD_{50}$ of IAV/PR8/34(H1N1), but all mice that were immunized with intranasal HA or saline died at day 10. About 80% of mice immunized twice with subcutaneous HA died at day 8 (Figure 3A). Even in more severe virus challenge, 90% of mice immunized with intranasal HA–SF–10 survived against infection with $160 \times LD_{50}$ of IAV/PR8/34(H1N1), while only 10% of mice immunized intranasally with HA-poly(I:C) and none of those immunized intranasally with HA or saline survived (Figure 3B).

Induction of HA-specific Th1- and Th2-type immune responses by HA–SF–10

To assess the effect of intranasal administration of HA–SF–10 on T-helper responses, we analyzed the subclasses of anti-HA-specific IgG (IgG1 and IgG2a) as well as IgE in sera and the induction of Th1- and Th2-type cytokine responses in mucosal lymphoid tissues. Intranasal immunization with HA–SF–10 induced not only anti-HA-specific IgG1 and Th2-type antibody, but also anti-HA-specific IgG2a and Th1-type antibody, and these levels suggested a balanced Th1/Th2 response (Figure 4A). The induced levels of IgG1 and IgG2a by intranasal administration of HA-SF-10 were significantly higher than those by HA-poly (I:C) ($P < 0{\cdot}01$ and $P < 0{\cdot}05$, respectively). However, the mean serum IgG2a/IgG1 ratio in mice immunized intranasally with HA–SF–10 was comparable with that of mice immunized with HA-poly(I:C) (Figure 4B). On the other hand, the mean serum IgG2a/IgG1 ratio was significantly low in mice immunized subcutaneously with HA compared with the value induced by HA–SF–10 ($P < 0{\cdot}05$).

Finally, we analyzed anti-HA-specific IgE (Figure 4C) and total IgE levels (Figure 4D) to exclude allergic responses after intranasal immunization with HA–SF–10. None of the mice immunized intranasally with HA–SF–10 showed the induction of anti-HA-specific IgE or total IgE in the serum, although distinct induction by subcutaneous administration of HA was detected. We also studied the expression levels of IFN-γ (Th1-associated cytokine) and IL-4 (Th2-associated cytokine) in mucosal lymphoid tissues of mice, such as NALT and nasopharynx, by ELISPOT (Figure 5). Mice immunized intranasally with HA–SF–10 showed significantly higher induction of both IL-4- and IFN-γ-producing cells than the induction of mice immunized intranasally with HA or saline. The numbers of induced IL-4- and IFN-γ-producing cells were higher in nasopharynx than those in NALT. The number of induced IL-4-producing cells and IFN-γ-producing cells supports the results of the balanced serum IgG2a/IgG1 ratio shown in Figure 4.

Discussion

Many potential mucosal adjuvants have been evaluated in the past, but no safe and efficacious mucosal adjuvant is available commercially at present. To overcome these issues, we recently reported a bovine pulmonary surfactant that enhances antigen delivery to dendritic cells, increases antigen sustainability in the nasal cavity, and induces both mucosal and systemic immunity.[9,14,15] To provide ample supply of this adjuvant without any risk of bovine spongiform encephalopathy, we prepared synthetic SSF, which comprises three major lipids, and synthetic human SP-C(1–35), resembling the natural surfactant.[9] Although the mucosal adjuvanticity of SSF is almost equivalent to that of natural Surfacten, there are four major problems that curtail the use

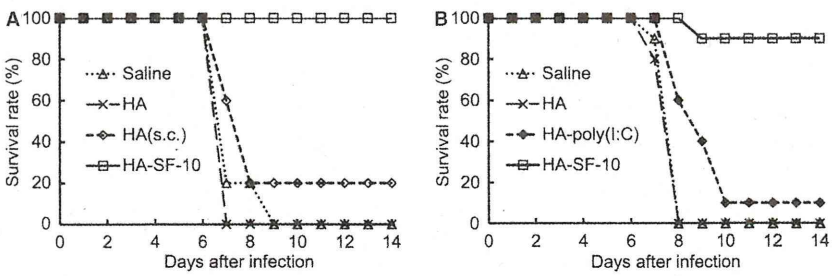

Figure 3. Survival rates of immunized mice after infection with lethal doses of IAV/PR8/34(H1N1) virus. (A) Mice were immunized with intranasal HA (0·2 μg) combined with or without SF-10 (2 μg), polyc (I:C) (2 μg) or saline three times every 2 weeks. Another group of mice received subcutaneous (s.c.) HA twice every two weeks. Two weeks after the last immunization, mice were infected with $10 \times LD_{50}$ (50 PFU) (A) and $160 \times LD_{50}$ (800 PFU) (B) of IAV/PR8/34(H1N1) virus, and their survival rates were monitored.

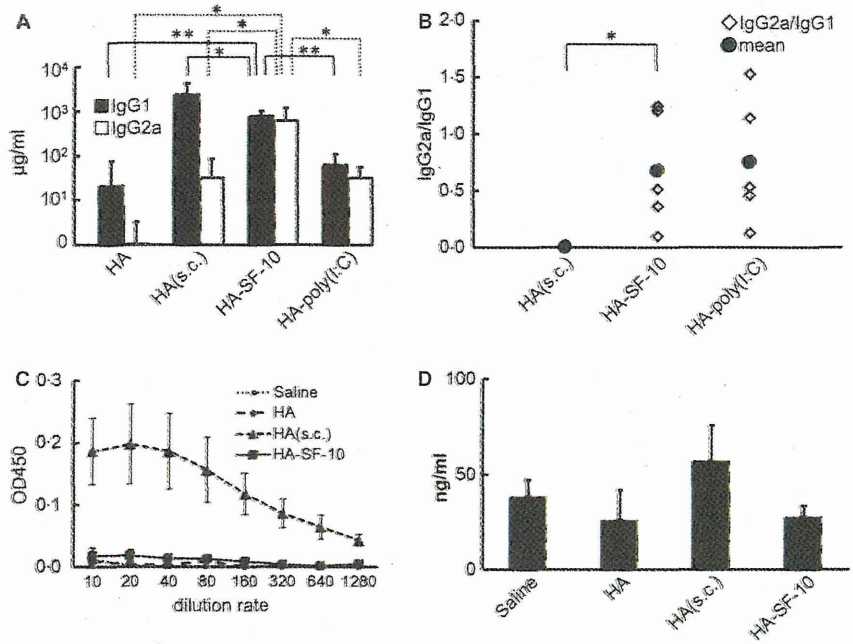

Figure 4. Induction of HA-specific Th1- and Th2-type immune responses by intranasal administration of HA–SF–10. Mice (n = 5) were immunized with intranasal HA (0·2 μg) combined with or without SF-10 (2 μg) or poly(I:C) (2 μg) three times every 2 weeks. Another group of mice received subcutaneous (s.c.) HA (0·2 μg) twice every 2 weeks. Two weeks after the last immunization, sera were collected, and anti-HA-specific IgG1, IgG2a, IgE, and total IgE levels were measured by ELISA. (A) Data are mean ± SD of IgG1 and IgG2a in sera. $*P < 0·05$, $**P < 0·01$. (B) Anti-HA-specific IgG2a/IgG1 ratio in sera of individual mice and their mean values. $*P < 0·05$. (C) Induced anti-HA-specific IgE levels in each animal group. Data (OD 450) are mean ± SD of each serum dilution rate. (D) Data are mean ± SD of total IgE concentrations in each animal group.

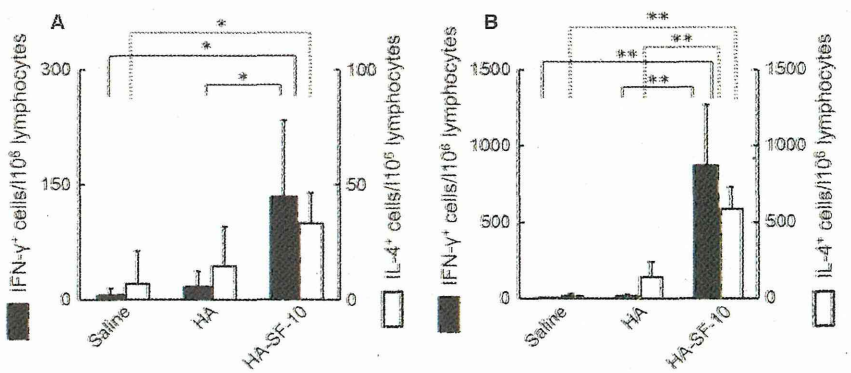

Figure 5. Induction of Th1- and Th2-type cytokines in airway lymphocytes by intranasal administration of HA–SF–10. Mice (n = 5) were immunized with intranasal HA (0·2 μg) combined with or without SF-10 (2 μg) or saline three times every 2 weeks. Two weeks after the last immunization, lymphocytes of NALT (A) and nasopharynx (B) were isolated and incubated with HA (5 μg/ml) for 16 hour. IFN-γ- and IL-4-producing lymphocytes were measured by ELISPOT. Data are mean ± SD of cytokine-producing lymphocytes per 10^6 lymphocytes in 4–5 independent experiments. $*P < 0·05$, $**P < 0·01$.

of SSF clinically: (i) Although SP-C(1–35) is an essential part of SSF, this hydrophobic peptide is insoluble in common organic solvents for large-scale manufacturing. In the present study, we identified a peptide from the SP-C-related peptides with stable adjuvanticity and solubility in methanol as a substitute for human SP-C(1–35). (ii) To further increase the antigen delivery efficacy of SSF, we increased HA binding to SSF at ≥92% by lyophilization. (iii) As HA and SSF mixing in a sonic oscillator is difficult to apply in industrial processing, we designed a processing method instead of sonication for large-scale manufacturing. (iv) As mucosal adjuvanticity of natural Surfacten or SSF was less potent than that of poly(I:C) (Table 2 and Figure 1), we increased the efficacy of SSF adjuvanticity by further modification.

In the present study, we solved four major problems of SSF stated above and developed a potent synthetic adjuvant,

SF-10. Among the active SP-C-related peptides (Tables 1 and 2), we selected K6L16 as a substitute for human SP-C(1–35), because K6L16 is soluble in methanol and expresses high adjuvanticity. To achieve more efficient interaction between HA and SSF and more efficient antigen delivery of SSF, they were mixed at 42°C, the critical temperature for surfactant lipids, for 10 minute, followed by lyophilization to remove water molecules between them, as a substitute for sonication. As shown in Table 3, lyophilization increased the binding of HA to SSF by ≥92% and markedly increased mucosal and systemic immunity. Lyophilization also seems to have two other benefits: protection against sonication-related loss of heat-labile HA antigenicity and large-scale manufacturing.

We added CVP to HA-SSF mixture at 0·5% to increase viscosity. The results showed the HA-SSF mixture prepared by lyophilization had higher adjuvanticity than that prepared by sonication (Table 3). The addition of CVP to the HA-SSF resulted in further increase in nasal-wash s-IgA production, probably due to the prolonged antigen presentation in the nasal cavity. Taken these improvements together, HA–SF–10 increased the induction of anti-HA-specific s-IgA in nasal washes and anti-HA-specific IgG in sera compared with intranasal HA-poly(I:C) and subcutaneous administration of HA (Figure 1). These data were supported by the neutralization activities in nasal washes, HI titers in serum, protective immunity, and high survival rates of animals immunized with intranasal HA–SF–10 (Figures 2 and 3, and Figure S1).

It has been reported that intranasal administration of the most potent toxin-based mucosal adjuvant of cholera toxin (CT) or *Escherichia coli* heat-labile enterotoxin (HLT) induces both nasal-wash s-IgA and serum IgG at about 3·8-fold of those by poly (I:C).[21] The data suggest that the efficacy of mucosal adjuvanticity of SF-10 is similar to that of CT and HLT, because nasal-wash s-IgA and serum IgG induced by HA–SF–10 were about 4-fold those induced by HA-poly (I:C) (Figure 1). Importantly, application of virosomal influenza vaccine adjuvanted with HLT resulted in high incidence of Bell's palsy[8] and CT-induced IgE against antigen and CT.[22] In contrast, SF-10 did not result in such adverse reactions in animal experiments. Intranasal mucosal live attenuated virus vaccines, FluMist® and FLUENZ, are currently available on the market. In a related issue, concern has been raised regarding the safety of FluMist® in young children aged<2 years with previous asthma or with recurrent wheezing.[11,12] Although not tested yet, HA–SF–10 influenza vaccine could be potentially useful in young children, because Surfacten® has been used in premature babies without significant adverse effects[13] and is known to enhance systemic and mucosal immunity in minipigs even just after weaning.[15]

For the development of effective and safe mucosal vaccine, it is important that the mucosal adjuvant induces a balanced Th1- and Th2-type cytokine response to support antigen-specific antibody production (Figure 4) without inflammatory or allergic side effects.[23,24] Figure 5 shows that intranasal immunization with HA–SF–10 elicited anti-HA-specific Th1 (IFN-γ)- and Th2(IL-4)-type cytokine responses in the nasopharynx and NALT, compared with HA and saline. HA–SF–10 also induced a balanced Th1- and Th2-type cytokine response in the airway mucosa. Of note, there was no detectable anti-HA-specific IgE and total IgE response in the sera of animals immunized intranasally with HA–SF–10. These results confirm that intranasal HA–SF–10 induces a balanced Th1- and Th2-type cytokine response without the risk of allergy.

IAV-specific $CD8^+$ cytotoxic T lymphocytes and IFN-γ-producing $CD4^+$ T lymphocytes promote clearance of IAV and recovery from infection.[5] Intranasal HA–SF–10 activated IFN-γ-producing lymphocytes in the nasopharynx and NALT and probably stimulated cellular immunity against IAV. Considered together, our results indicate that intranasal immunization with HA–SF–10 provided efficient protection against IAV infection and markedly increased survival rates even in mice with fulminant viral infection. Administration of antigen-SF-10 by other mucosal routes should be evaluated in future studies.

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Conflict of interest

HK and DM are inventors of an applied patent related to the technology described in this study (Preparation methods of SF-10 adjuvant; application number WO 2011/108521 A1), which is owned by the University of Tokushima. All remaining authors declare no conflict of interest.

References

1 Demicheli V, Jefferson T, Rivetti D, Deeks J. Prevention and early treatment of influenza in healthy adults. Vaccine 2000; 18:957–1030.
2 Jefferson T, Rivetti D, Rivetti A, Rudin M, Di Pietrantonj C, Demicheli V. Efficacy and effectiveness of influenza vaccines in elderly people: a systematic review. Lancet 2005; 366:1165–1174.
3 Clements ML, Betts RF, Tierney EL, Murphy BR. Serum and nasal wash antibodies associated with resistance to experimental challenge with influenza A wild type virus. J Clin Microbiol 1986; 24:157–160.
4 Holmgren J, Czerkinsky C. Mucosal immunity and vaccines. Nat Med 2005; 11:45–53.
5 Doherty PC, Topham DJ, Tripp RA, Cardin RD, Brooks JW, Stevenson PG. Effector $CD4^+$ and $CD8^+$ T-cell mechanisms in the control of respiratory virus infections. Immunol Rev 1997; 159:105–117.

6 Briscoe JH. Intranasal immunization with inactivated influenza virus vaccine in a boys' boarding school. Practitioner 1975; 214:821–826.
7 Davis SS. Nasal vaccines. Adv Drug Deliv Rev 2001; 51:21–42.
8 Mutsch M, Zhou W, Rhodes P *et al.* Use of the inactivated intranasal influenza vaccine and the risk of Bell's palsy in Switzerland. N Engl J Med 2004; 350:896–903.
9 Mizuno D, Kimoto T, Takei T *et al.* Surfactant protein C is an essential constituent for mucosal adjuvanticity of Surfacten, acting as an antigen delivery vehicle and inducing both local and systemic immunity. Vaccine 2011; 29:5368–5378.
10 Belshe RB, Nichol KL, Black SB *et al.* Safety, efficacy, and effectiveness of live, attenuated, cold-adapted influenza vaccine in an indicated population age 5–49 years. Clin Infect Dis 2004; 39:920–927.
11 Belshe RB, Edwards KM, Vesikari T *et al.* Live attenuated versus inactivated influenza vaccine in infants and young children. N Engl J Med 2007; 356:685–696.
12 Esposito S, Montinaro V, Groppali E, Tenconi R, Semino M, Principi N. Live attenuated intranasal influenza vaccine. Hum Vaccin Immunother 2012; 8:17–20.
13 Konishi M, Chida S, Shimada S *et al.* Surfactant replacement therapy in premature babies with respiratory distress syndrome: factors affecting the response to surfactant and comparison of outcome from 1982–86 and 1987–91. Acta Paediatr Jpn 1992; 34:617–630.
14 Mizuno D, Ide-Kurihara M, Ichinomiya T, Kubo I, Kido H. Modified pulmonary surfactant is a potent adjuvant that stimulates the mucosal IgA production in response to the influenza virus antigen. J Immunol 2006; 176:1122–1130.
15 Nishino M, Mizuno D, Kimoto T *et al.* Influenza vaccine with Surfacten, a modified pulmonary surfactant, induces systemic and mucosal immune responses without side effects in minipigs. Vaccine 2009; 27:5620–5627.
16 Wright JR, Clements JA. Metabolism and turnover of lung surfactant. Am Rev Respir Dis 1987; 136:426–444.
17 Baritussio A, Pettenazzo A, Benevento M, Alberti A, Gamba P. Surfactant protein C is recycled from the alveoli to the lamellar bodies. Am J Physiol 1992; 263:607–611.
18 Takahashi E, Kataoka K, Fujii K *et al.* Attenuation of inducible respiratory immune responses by oseltamivir treatment in mice infected with influenza A virus. Microbes Infect 2010; 12:778–783.
19 Asanuma H, Inaba Y, Aizawa C, Kurata T, Tamura S. Characterization of mouse nasal lymphocytes isolated by enzymatic extraction with collagenase. J Immunol Methods 1995; 187:41–51.
20 Ichinohe T, Watanabe I, Ito A *et al.* Synthetic double-strand RNA poly (I :C) combined with mucosal vaccine protects against influenza virus infection. J Virol 2005; 79:2910–2919.
21 Asahi-Ozaki Y, Itamura S, Ichinohe T *et al.* Intranasal administration of adjuvant-combined recombinant influenza virus HA vaccine protects mice from the lethal H5N1 virus infection. Microbes Infect 2006; 8:2706–2714.
22 Tamura S, Shoji Y, Hasiguchi K, Aizawa C, Kurata T. Effects of cholera toxin adjuvant on IgE antibody response to orally or nasally administered ovalbumin. Vaccine 1994; 12:1238–1240.
23 Trumpfheller C, Caskey M, Nchinda G *et al.* The microbial mimic poly IC induces durable and protective $CD4^+$ T cell immunity together with a dendritic cell targeted vaccine. Proc Natl Acad Sci USA 2008; 105:2574–2579.
24 Tantilipikorn P, Auewarakul P. Airway allergy and viral infection. Asian Pac J Allergy Immunol 2011; 29:113–119.

Supporting Information

Additional Supporting Information may be found in the online version of this article:

Figure S1. HI activity in sera of mice immunized with intranasal administration of HA–SF–10, HA and saline and with subcutaneous administration of HA.

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Blood Lactate/ATP Ratio, as an Alarm Index and Real-Time Biomarker in Critical Illness

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Abstract

Objective: The acute physiology, age and chronic health evaluation (APACHE) II score and other related scores have been used for evaluation of illness severity in the intensive care unit (ICU), but there is still a need for real-time and sensitive prognostic biomarkers. Recently, alarmins from damaged tissues have been reported as alarm-signaling molecules. Although ATP is a member of the alarmins and its depletion in tissues closely correlates with multiple-organ failure, blood ATP level has not been evaluated in critical illness. To identify real-time prognostic biomarker of critical illness, we measured blood ATP levels and the lactate/ATP ratio (ATP-lactate energy risk score, A-LES) in critically ill patients.

Methods and Results: Blood samples were collected from 42 consecutive critically ill ICU patients and 155 healthy subjects. The prognostic values of blood ATP levels and A-LES were compared with APACHE II score. The mean ATP level (SD) in healthy subjects was 0.62 (0.19) mM with no significant age or gender differences. The median ATP level in severely ill patients at ICU admission was significantly low at 0.31 mM (interquartile range 0.25 to 0.44) than the level in moderately ill patient at 0.56 mM (0.38 to 0.70) ($P<0.01$). Assessment with ATP was further corrected by lactate and expressed as A-LES. The median A-LES was 2.7 (2.1 to 3.3) in patients with satisfactory outcome at discharge but was significantly higher in non-survivors at 38.9 (21.0 to 67.9) ($P<0.01$). Receiver operating characteristic analysis indicated that measurement of blood ATP and A-LES at ICU admission are as useful as APACHE II score for prediction of mortality.

Conclusion: Blood ATP levels and A-LES are sensitive prognostic biomarkers of mortality at ICU admission. In addition, A-LES provided further real-time evaluation score of illness severity during ICU stay particularly for critically ill patients with APACHE II scores of $\geq$20.0.

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Competing Interests: The authors have declared that no competing interests exist.

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Introduction

The recent years have witnessed a wide used of the acute physiology, age and chronic health evaluation (APACHE) II score [1], the simplified acute physiology score (SAPS) II [2] and other related scoring systems [3,4] in the evaluation of the severity of illness in the intensive care unit (ICU). These scoring systems have several drawbacks, in particular their time-consuming evaluation, some unavailable data from automatic equipments and inter-examiner discrepancies [5]. Thus, these scores are not always utilized in daily practice of many ICUs [6].

Adenosine 5′-triphosphate (ATP) is the "energy currency" of organisms and plays central roles in bioenergetics, whereby its level is used to evaluate cell viability and proliferation [7–10], cell death [11,12], and energy transmission [13]. Human typically uses about body weight of ATP over the course of the day [14]. In addition, ATP release from damaged cells and tissues has recently attracted attention, and has been reported as an alarm signal compound, alarmin [15,16]. Alarmins were originally categorized as endogenous damage-associated molecular pattern (DAMP) molecules, to separate them from exogenous pathogen-associated molecular pattern (PAMP) molecules [17], associated with overstimulation of the immune system [18]. The released ATP in serum, however, is rapidly degraded within few minutes [19] and the levels are difficult to evaluate correctly. Therefore, we can only monitor ATP levels in blood cells, as the net value of intracellular ATP production and ATP degradation and/or release.

The three main pathways to generate ATP in eukaryotic organisms are glycolysis, the citric acid cycle/oxidative phosphorylation and fatty acid β-oxidation. Once ATP generation in the mitochondria is impaired in various diseases, energy source metabolites, such as carbohydrate metabolites and fatty acid metabolites are converted to and stored as lactate and ketone bodies, respectively. In fact, hyperlactatemia develops in nearly half of patients admitted to the ICU, and presentation with or development of hyperlactatemia is associated with a significant

increase in the incidence of organ failure, metabolic dysfunction and mortality [20]. To find real-time and reliable biomarker(s) for the progression state of critical illness, we evaluated blood ATP levels in combination with serum lactate levels as a new alarm reporter in critical illness and the values were compared with the APACHE II score.

Critically ill patients with multiple organ failure (MOF) and septic non-survivors show a decrease in mitochondrial activity and ATP production, and increase in lactate concentrations in leg muscles [21–23]. In addition, we recently demonstrated that influenza A virus infection triggers MOF and acute myocarditis with ATP depletion in mice as well as impairment of mitochondrial membrane potential in cardiomyoblasts [24]. Blood ATP depletion was also identified in children with influenza-associated acute encephalopathy and in patients with mitochondrial diseases [25].

In the measurement of ATP levels in various tissues and blood, we recently found that the chaotropic ATP extraction reagents recommended in the commercially available assay kits so far (e.g., trichloroacetic acid, perchloric acid and ethylene glycol), are useful only for materials with relatively low protein concentrations, but not suitable for tissues with high protein concentrations. ATP is co-precipitated with insoluble protein during homogenization in high protein concentrations. Accordingly, we improved the ATP extraction efficiency from tissues and cells using a novel phenol-based extraction reagent [26].

The present study was designed to determine the control gender-specific blood ATP levels measured in healthy individuals of various ages, using our highly reliable extraction method. We then used the "normal" blood ATP levels of the control to evaluate the blood ATP levels and lactate (mM)/ATP (mM) ratio (expressed as the ATP-lactate energy risk score, A-LES) in patients admitted to the ICU. The A-LES was used as a real-time prognostic alarm biomarker and the values were compared with the APACHE II scores.

Table 1. Whole blood ATP, lactate and A-LES levels in healthy subjects.

Age (years)	Sex	Lactate (mM)	ATP (mM)	A-LES
0 to 19	Males (*n* = 6)	1.43±1.07	0.71±0.11	1.50±1.46
	Females (*n* = 7)	<1.69±1.06	0.64±0.13	<2.88±1.70
20 to 29	Males (*n* = 11)	1.48±0.38	0.79±0.18	1.99±0.61
	Females (*n* = 10)	1.03±0.25	0.71±0.20	1.40±0.48
30 to 39	Males (*n* = 15)	<1.38±0.47	0.68±0.11	<2.05±0.84
	Females (*n* = 8)	1.20±0.25	0.79±0.19	1.60±0.47
40 to 49	Males (*n* = 12)	1.46±0.39	0.83±0.25	1.90±0.73
	Females (*n* = 12)	<1.05±0.35	0.67±0.17	<1.63±0.60
50 to 59	Males (*n* = 9)	<1.38±0.48	0.64±0.19	<2.17±0.45
	Females (*n* = 4)	<0.81±0.03	0.52±0.12	<1.61±0.32
60 to 69	Males (*n* = 8)	<1.05±0.27	0.52±0.10	<2.04±0.54
	Females (*n* = 11)	<0.81±0.03	0.47±0.05	<1.73±0.18
70 to 92	Males (*n* = 13)	<0.99±0.23	0.47±0.06	<2.14±0.62
	Females (*n* = 29)	<1.00±0.36	0.46±0.05	<2.18±0.73
0 to 92	Males (*n* = 74)	<1.31±0.49	0.66±0.20	<2.05±0.73
	Females (*n* = 81)	<1.08±0.49	0.57±0.17	<1.96±0.91
	Total (*n* = 155)	<1.19±0.50	0.62±0.19	<2.00±0.83

Data are mean±SD. *n* = number of healthy subjects. Blood lactate levels below the limit of measurement (<0.8 mM) are reported as <0.8 mM.
doi:10.1371/journal.pone.0060561.t001

Materials and Methods

Ethics Statement

For clinical studies, written informed consent was obtained directly from each study participant or their legal representative before enrolment. Also, all healthy individuals gave assent if able to understand, and their parents or guardians gave written informed consent and permission to participate in this study. Permission to perform scientific studies and ethical approval of the study protocol were granted the Ethics Committee of Tokushima University Hospital (Permit Number: #901). The study was conducted under the supervision of the physicians involved (MN, RO, MO, EN, KS and MM), and patients were advised of risks, benefits and the right to withdraw from further involvement in the study at any point without repercussions. All data, particularly patient identifying data, were physically and electronically secured throughout the study.

Patients

We evaluated 42 consecutive patients admitted to ICU from November 2009 to November 2010. All patients received early goal-directed therapy according to a standard protocol that emphasized adequate volume administration, appropriate therapeutic drug administration, and optimal oxygen delivery. The study also included 155 healthy individuals free from any acute or chronic illness.

Blood Collection

Blood was withdrawn from the antecubital vein of healthy individuals into either Vacutainer tubes (BD vacutainer; Becton Dickinson Diagnostics, Tokyo, Japan) or syringes containing either ethylenediaminetetraacetic acid (EDTA) or sodium heparin. For ICU patients, the blood samples were usually collected from the arterial line into EDTA vacutainers, but in some cases collected from the antecubital vein or central vein. In each patient, blood was sampled at serial time points during the ICU stay. After withdrawal of 5.0 mL of arterial/venous blood, the sample was transferred to a 15.0-mL Falcon tube. Blood gas data, such as PO_2 and PCO_2, and data of total hemoglobin (tHb), blood glucose (BG), and lactate were monitored by a blood gas analyzer (Blood Gas System 860; Bayer Diagnostics, Tokyo, Japan). Blood aliquots (0.1 mL) were added to 1.3 mL of Tris-EDTA-saturated phenol (phenol-TE) ATP extraction reagents (AMERIC-ATP kit; Wako Pure Chemical Industries, Osaka, Japan), thoroughly shaken for 20 seconds and then stored at −20°C until use.

Measurement of Blood ATP

Blood ATP levels were measured by the firefly bioluminescence assay kit (AMERIC-ATP kit; Wako Pure Chemical Industries, Osaka, Japan) according to the protocol supplied by the manufacturer or as described previously [26]. Briefly, the extracted blood sample was shaken and centrifuged (10,000 × *g*, 5 minutes at 4°C) to achieve phase separation; 50 μL of the upper aqueous phases was diluted 10,000-fold with deionized water. Then 10 μL of this diluted extract was injected into 90 μL of luciferin/luciferase mixture, and the bioluminescence product was immediately measured by a luminometer (GloMax-96 Microplate Luminometer; Promega, Tokyo, Japan). Blood ATP level (mM) in each sample was calculated from the calibration curve.

Table 2. Comparison of blood lactate and ATP levels in radial arterial (A), pulmonary arterial (PA), and central venous (CV) blood.

Patients no./Time (h)*	Lactate (mM)			ATP (mM)			A-LES		
	A	PA	CV	A	PA	CV	A	PA	CV
07/0	1.79	1.69	1.97	0.70	0.72	0.73	2.56	2.35	2.70
07/3	1.95	1.97	2.16	0.83	0.83	0.80	2.35	2.37	2.70
07/6	2.09	2.01	2.01	0.34	0.35	0.34	6.15	5.74	5.91
07/24	1.68	1.69	1.63	2.88	2.88	2.76	2.47	2.49	2.60
08/3	2.88	2.88	2.76	0.96	0.99	0.98	3.00	2.91	2.82
08/6	2.47	2.49	2.60	0.55	0.53	0.55	4.49	4.70	4.73
09/0	2.29	2.43	2.73	0.36	0.40	0.37	6.36	6.08	7.38
10/3	1.64	–	1.51	0.33	–	0.34	4.97	–	4.44
18/0	6.10	5.48	5.75	0.42	0.41	0.41	14.52	13.37	14.02
19/0	5.39	5.72	5.91	0.35	0.38	0.38	15.40	15.03	15.55
21/0	1.47	1.30	1.37	0.53	0.57	0.53	2.77	2.28	2.58

*Time period (h, hours) after ICU admission.
doi:10.1371/journal.pone.0060561.t002

Calculation of APACHE II Score and A-LES

The severity of illness was evaluated in each patient within the first 24 hours of ICU admission using the APACHE II score [1,27]. The score was also determined every 24 hours during the ICU stay. The A-LES score, representing [serum lactate (mM)/blood ATP (mM)], was determined for each patient during the ICU stay.

Table 3. Patient demographics and clinical findings.

Patient demographics	
n	42
Age, years	68 (34 to 79)
Sex, % male:% female	52:48
First 24-h APACHE II score	18 (13 to 25)
Duration of ICU stay, days	3 (1 to 10)
Diagnosis at admission	No. of patients (%)
Post-cardiovascular surgery	9 (21.4)
Septic shock	7 (16.7)
Acute coronary syndrome	4 (9.5)
Congestive heart failure	4 (9.5)
Infective endocarditis	3 (7.1)
Liver cirrhosis	3 (7.1)
Pneumonia	2 (4.8)
Interstitial pneumonia	2 (4.8)
Stroke	2 (4.8)
Others	6 (14.3)
Blood biochemical tests*	
Glucose, mg/dL	155 (125 to 227)
Lactate, mM	2.72 (1.92 to 5.78)
ATP, mM	0.38 (0.29 to 0.56)
A-LES	8.00 (4.74 to 13.37)
Hemoglobin, g/dL	10.4 (9.1 to 11.1)
Leukocyte count,/μL	12,700 (9,300 to 17,100)
Platelet count, x1,000/μL	114 (46 to 182)

Data are median (interquartile range) and number of patients (%).
*Data represent results of analysis of samples taken at admission to the ICU.
doi:10.1371/journal.pone.0060561.t003

Statistical Analysis

ICU patients were divided into two groups based on the severity of illness on admission: moderately ill patients (APACHE II score <20) and severely ill patients (APACHE II score ≥ 20). The outcome of the patients was divided into two categories: survival and non-survival. Data were analyzed for statistical significance across groups using nonparametric Mann-Whitney's U test. Correlations were calculated by determining Spearman's rank correlation coefficient (r_s). P values less than 0.05 were considered statistically significant. Receiver operating characteristic (ROC) curves were constructed using Microsoft Excel software (Microsoft Corporation, Redmond, WA) add-in Ekuseru-Toukei 2010 version 1.10 (Social Survey, Research Information Co.) to evaluate the accuracy of risk prediction comparing the calculated mortality with the actual deaths.

Results

ATP, Lactate and A-LES Values in Healthy Subjects

At the beginning of the study, we determined the levels of ATP by the new phenol-TE extraction method [26] and lactate in venous blood samples from 155 healthy males and females (age, range 0 to 92 years). The measured levels showed normal distribution pattern, with a mean (SD) value of 0.62 mM (0.19) (Table 1). There was no significant sex difference in ATP level. The ATP levels tended to be slightly lower in subjects aged ≥ 60 years than those in younger subjects, although the difference was not significant. The mean blood lactate level under resting-state condition was <1.19 mM (<0.50) in the healthy group, with no significant age or gender difference. The mean A-LES (SD) was <2.00 (0.83) with no significant age or gender difference in the control subjects.

Similar ATP and Lactate Levels in Arterial and Central Venous Blood

Table 2 shows the data of ATP, lactate and A-LES levels in arterial and central venous blood of 7 representative ICU patients.

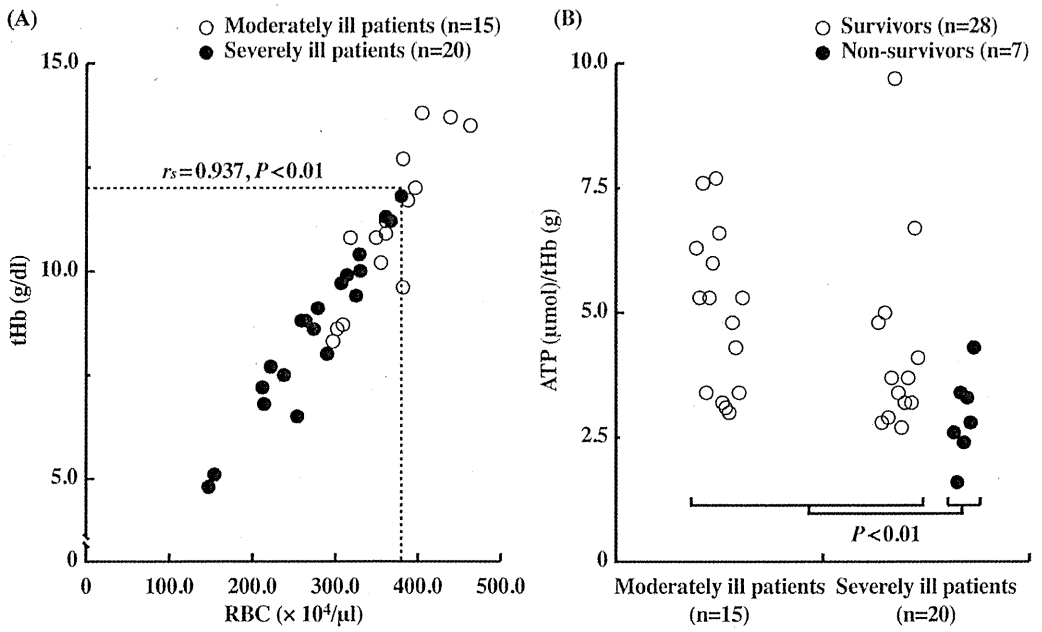

Figure 1. Blood ATP levels correlate with RBC count and ATP levels in ICU patients. (A) Correlation between tHb (total hemoglobin) and RBC count in the available data of moderately (n = 15) and severely ill patients (n = 20) during the ICU stay. The *Dotted lines* represent the lower limits of tHb and RBC count of healthy subjects. (○): moderately ill patients, (●) severely ill patients. (B) ATP concentration in whole blood, expressed as micromoles ATP per gram total hemoglobin (μmol/g tHb) in ICU patients. (○): survivors, (●) non-survivors. The values of ATP/tHb in non-survivors were significantly lower than those in survivors (P<0.01).
doi:10.1371/journal.pone.0060561.g001

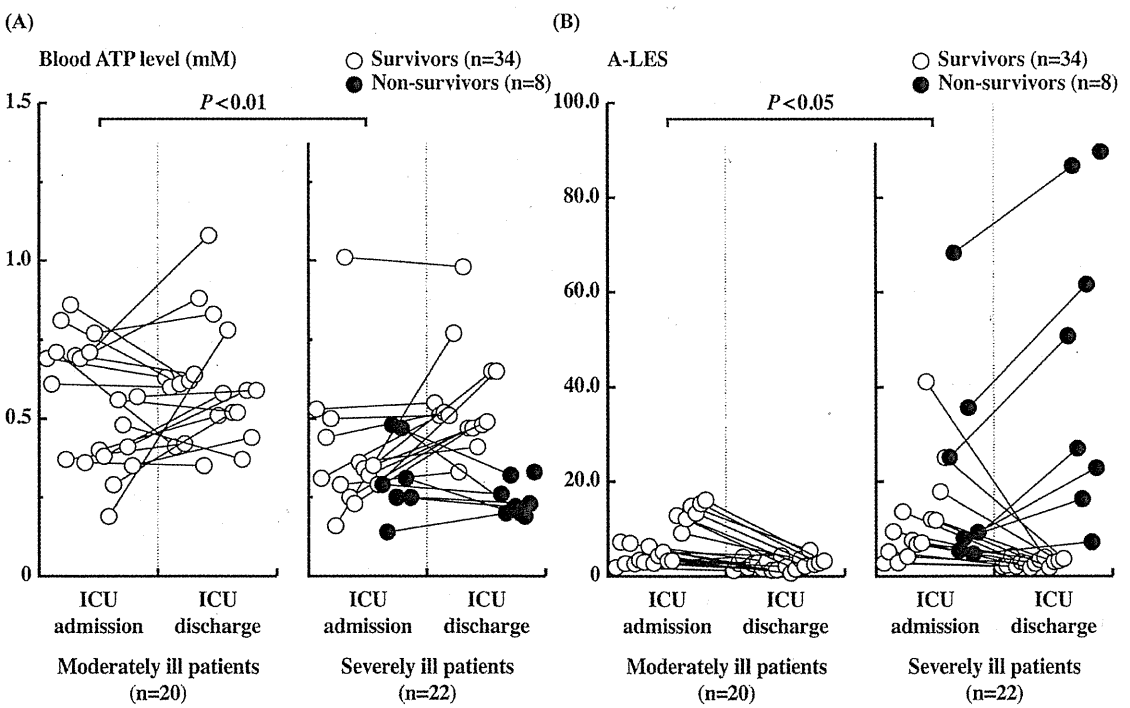

Figure 2. Changes in blood ATP and A-LES levels in moderately and severely ill patients. (A) Blood ATP levels and (B) A-LES values at ICU admission and ICU discharge. Symbols are paired data of individual patients. (○): survivors, (●) non-survivors. Levels of ATP and A-LES at ICU admission in severely ill patients were significantly lower than those in moderately ill patients (P<0.01 for ATP and P<0.05 for A-LES).
doi:10.1371/journal.pone.0060561.g002

Table 4. Comparison of changes in blood ATP level, A-LES, and APACHE II score during the course of ICU stay.

		ICU admission	ICU discharge
Moderately ill patients ($n=20$)	ATP, mM	0.56 (0.38 to 0.70)	0.53 (0.44 to 0.61)
	A-LES	6.7 (3.1 to 12.4)	2.7 (1.8 to 3.2)
	APACHE II score	12.5 (9.0 to14.3)	11.5 (9.0 to 13.3)
Severely ill patients ($n=22$)	ATP, mM	0.31 (0.25 to 0.44)**	0.45 (0.28 to 0.52) *
	A-LES	9.5 (6.8 to 17.9)*	3.6 (2.7 to 21.0) **
	APACHE II score	25.0 (20.0 to 31.0)**	21.0 (16.5 to 25.0)**
Septic shock patients ($n=7$)	ATP, mM	0.29 (0.27 to 0.33)**	0.48 (0.33 to 0.57)
	A-LES	12.1 (10.7 to 19.4)*	3.1 (2.9 to 9.6) **
	APACHE II score	28.0 (22.0 to 36.0)**	15.0 (14.0 to 19.0)**
Survivors ($n=34$)	ATP, mM	0.41 (0.33 to 0.60)	0.52 (0.45 to 0.62)
	A-LES	7.4 (3.7 to 12.7)	2.7 (2.1 to 3.3)
	APACHE II score	15.5 (11.3 to 22.3)	13.5 (11.0 to 16.0)
Non-survivors ($n=8$)	ATP, mM	0.29 (0.25 to 0.39)#	0.23 (0.20 to 0.28)##
	A-LES	9.3 (6.7 to 30.4)#	38.9 (21.0 to 67.9)##
	APACHE II score	25.0 (20.0–31.0)##	21.0 (16.5 to 25.0)##
Total ($n=42$)	ATP, mM	0.38 (0.29 to 0.56)	0.51 (0.36 to 0.59)
	A-LES	8.0 (4.7 to 13.4)	3.0 (2.1 to 4.2)
	APACHE II score	19.0 (13.0 to 25.0)	15.0 (12.3 to 21.5)

Data are median (interquartile range). n = number of healthy subjects.
*$P<0.05$,
**$P<0.01$ versus moderately ill patients: Mann-Whitney's U-test.
#$P<0.05$,
##$P<0.01$ versus survivors: Mann-Whitney's U-test.
doi:10.1371/journal.pone.0060561.t004

Regression analysis to validate the correlation among ATP, lactate and A-LES levels in arterial and central venous blood showed almost perfect correlation with high correlation coefficients: $r_s = 1.00$ for ATP, $r_s = 0.98$ for lactate and $r_s = 1.00$ for A-LES between blood collected from radial and pulmonary arteries ($P<0.001$); and $r_s = 1.00$ for ATP, $r_s = 0.97$ for lactate and $r_s = 0.99$ for A-LES between radial artery and central venous blood ($P<0.001$). The results were consistent with the previous report of equivalent lactate levels in blood samples from peripheral vein, pulmonary artery and central vein [28].

ATP, Lactate Levels and A-LES in ICU Patients

Next, we measured the levels of blood ATP and serum lactate and calculated the A-LES in 42 patients admitted to the ICU. The levels of these parameters showed skewed distribution. Table 3 lists the demographic data while Tables S1, S2 and S3 list the individual data and clinical characteristics. The major diagnosis on admission was post-cardiovascular surgery (21.4%), followed by septic shock (16.7%). The median values (interquartile range) of ATP, lactate and A-LES were 0.38 mM (0.29 to 0.56), 2.72 mM (1.92 to 5.78 mM) and 8.00 (4.74 to 13.37), respectively, and the levels were all within the abnormal range compared to those in healthy subjects (Table 1).

Blood ATP Levels Normalized by Total Hemoglobin in ICU Patients

Blood cells are diluted by transfusion and low red blood cell (RBC) count is usually found in patients with advanced disease, resulting in low blood ATP levels in severely ill patients. Since the major source of ATP in blood is RBC, we determined RBC count and total hemoglobin (tHb) in moderately ill patients (APACHE II score <20) and severely ill patients (APACHE II score ≥20) during ICU admission. The RBC count correlated with tHb level ($r_s = 0.937$, $P<0.01$), and was significantly lower in severely ill patients than moderately ill patients ($P<0.01$) (Figure 1A). Blood ATP levels normalized by tHb levels (the ATP/tHb) were significantly lower in non-survivors than those in survivors ($P<0.01$) (Figure 1B).

Changes in Blood ATP and A-LES Levels in Critically Ill Patients

To identify a sensitive and real-time prognostic biomarker of critical illness, we evaluated the levels of ATP and A-LES in 42 patients during critical illness (Figure 2). ATP levels at ICU admission were significantly lower in severely ill patients than in moderately ill patients ($P<0.01$) (Figure 2A): the median ATP level was 0.31 mM (0.25 to 0.44) in severely ill patients and 0.56 mM (0.38 to 0.70) in moderately ill patients at ICU admission (Table 4). Notably, the median ATP level of 7 patients with septic shock on admission was low at 0.29 mM (0.27 to 0.33), which was significantly lower ($P<0.01$) than the level in moderately ill patients (Tables 4 and S2). The ATP levels generally recovered during ICU stay in large numbers of survivors. Furthermore, the median ATP level in non-survivors [0.23 mM (0.20 to 0.28)] was significantly lower than that of survivors [0.52 mM (0.45 to 0.62)] at ICU discharge ($P<0.01$) (Table 4).

In contrast to the changes in blood ATP levels during ICU admission, the change in A-LES was clearer particularly in severely ill patients and non-survivors (Figure 2B). The A-LES decreased in all survivors in both moderately and severely ill patients without exception and the median A-LES of survivors was 2.7 (2.1 to 3.3) at ICU discharge (Table 4). In contrast, the median

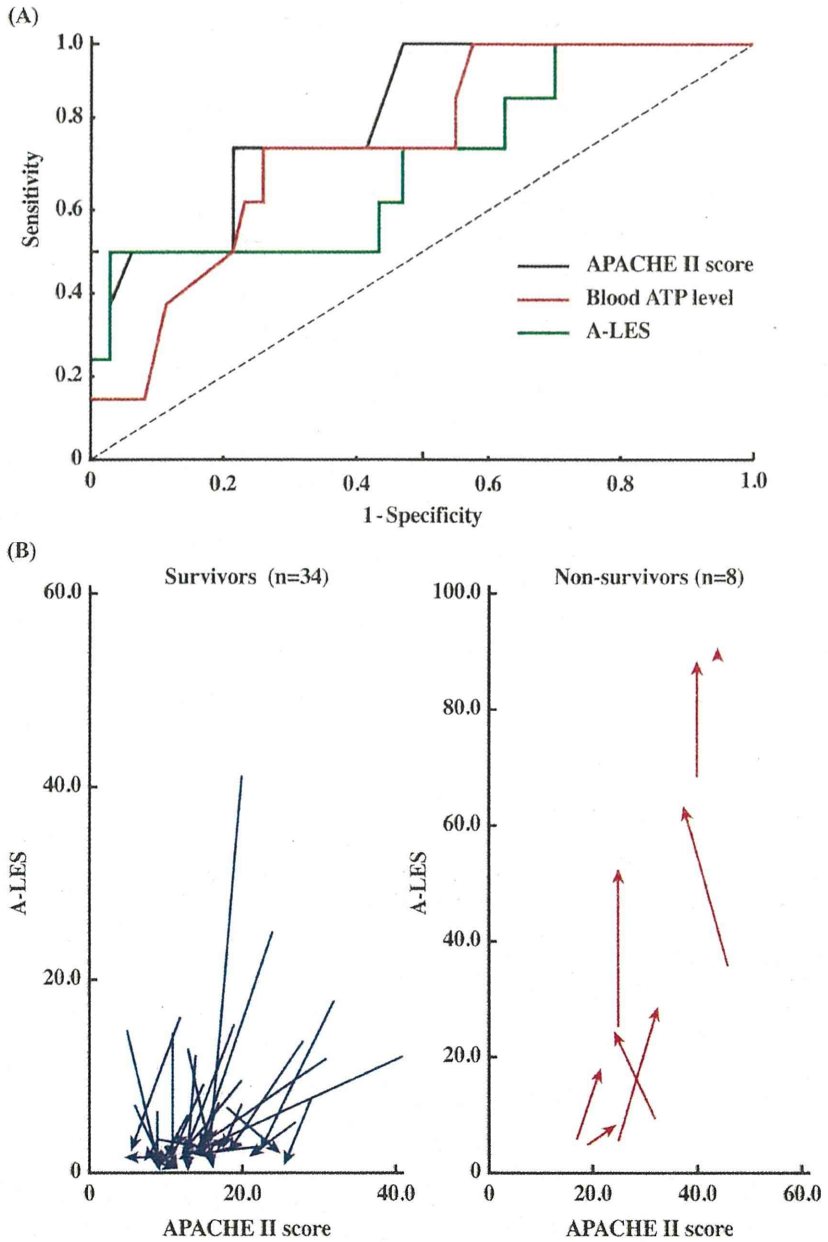

Figure 3. Relationship between APACHE II score and A-LES. (A) ROC analysis in prediction of mortality at the time of ICU admission. Dotted diagonal line = no discrimination. (B) Correlation between changes in APACHE II score and A-LES score during ICU stay in survivors and non-survivors. Data are values measured at two time points: at initial ICU admission and ICU discharge. Arrows indicate from ICU admission to discharge.
doi:10.1371/journal.pone.0060561.g003

A-LES in non-survivors at ICU discharge was 38.9 (21.0 to 67.9), which was significantly higher than the value of survivors ($P<0.01$) (Table 4). These results indicate that A-LES is a highly sensitive prognostic marker of critical illness.

Evaluation of A-LES as a Prognostic Marker and Correlation with APACHE II in ICU Patients

To evaluate A-LES and ATP levels of patients at the time of ICU admission for prediction of mortality, ROC analysis was performed (Figure 3A and Table 5). The values of the area under ROC curve (AUC) for APACHE II, ATP and A-LES were of similar range (>0.5) and measured 0.83, 0.75 and 0.71, respectively, indicating that ATP level and A-LES are as useful as APACHE II score for prediction of mortality.

Figure 3B shows changes in A-LES and APACHE II scores measured during ICU admission. Although APACHE II scores did not sensitively express the change in the critical state of ICU patients, particularly patients with severe illness (APACHE II range, ≥20.0), A-LES reflected well the change in the critical state. Markedly high A-LES values (up to 89.7) in non-survivors and low values in all survivors were observed during ICU admission. These

Table 5. ROC analysis for prediction of mortality in 42 patients at ICU admission.

Variable	AUC	Cut-off value	Sensitivity/specificity (%)
APACHE II score	0.83	>20	71/59
A-LES	0.71	>20	43/94
Blood ATP levels*	0.75	>3	71/74

*The reciprocal of blood ATP levels was used for ROC analysis.
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results indicate that A-LES does not only predict mortality at the time of ICU admission in a manner similar to APACHE II, but also provides a sensitive evaluation score of change in illness severity during ICU admission. Figure 4 illustrates the changes in blood ATP, A-LES and APACHE II during ICU admission of representative patients of the three groups (moderately ill patients on admission and discharge, severely ill patients on admission and discharge, and severely ill patients on admission who died during admission). Among the three parameters, A-LES provided the best prognostic information; almost all patients with satisfactory outcome at discharge had A-LES of <5.49, while A-LES during admission was >20.0 in non-survivors.

Discussion

Risk prediction is an important issue in intensive care. The APACHE II score is used as a severity score during the first 24 hours of ICU admission while the SOFA score was developed to estimate morbidity during ICU stay. Although several clinical and laboratory parameters have been evaluated for the prediction of mortality during ICU stay, real-time and easily measurable prognostic biomarker(s) are desirable. During critical illness, the serum contains various PAMP molecules, particularly in severe infection [29], DAMP molecules released by stressed or damaged tissues [17] and host cellular response molecules with regulatory function against these PAMP and DAMP molecules. Among the molecules, alarm reporter(s) might be an important prognostic value. Recently, ATP released from damaged tissues has been classified as a danger signal, alarmin, which induces proinflammatory cytokines, but it is rapidly degraded within few minutes by ecto-ATPase [19]. Although released ATP cannot be analyzed accurately, cellular ATP levels, including blood cell ATP levels, can be measured easily, representing the sum of ATP production and ATP degradation and/or release.

We recently established an efficient and improved phenol-based ATP extraction method instead of the chaotropic extraction reagents recommended in the commercially available assay kit, which involves co-precipitation of ATP with insoluble proteins during homogenization and extraction [26]. In the present study, we measured blood ATP levels by a phenol-based extraction reagent and reported the "normal" blood ATP levels in 155 healthy individuals ranging in age from 0 to 92 years. The mean blood ATP level (SD) in healthy subjects was 0.62 (0.19) mM. Age and gender had no significant effect on ATP level, although the values tended to decrease with advancing age, particularly over 60 years of age, and values in males were slightly higher than those in females with some exceptions, probably because of age and gender differences in the number of red blood cells (Table 1).

The present study established the clinical utility of a sensitive and real-time alarm index, A-LES, which consists of ATP and lactate. The median A-LES of patients with satisfactory outcome

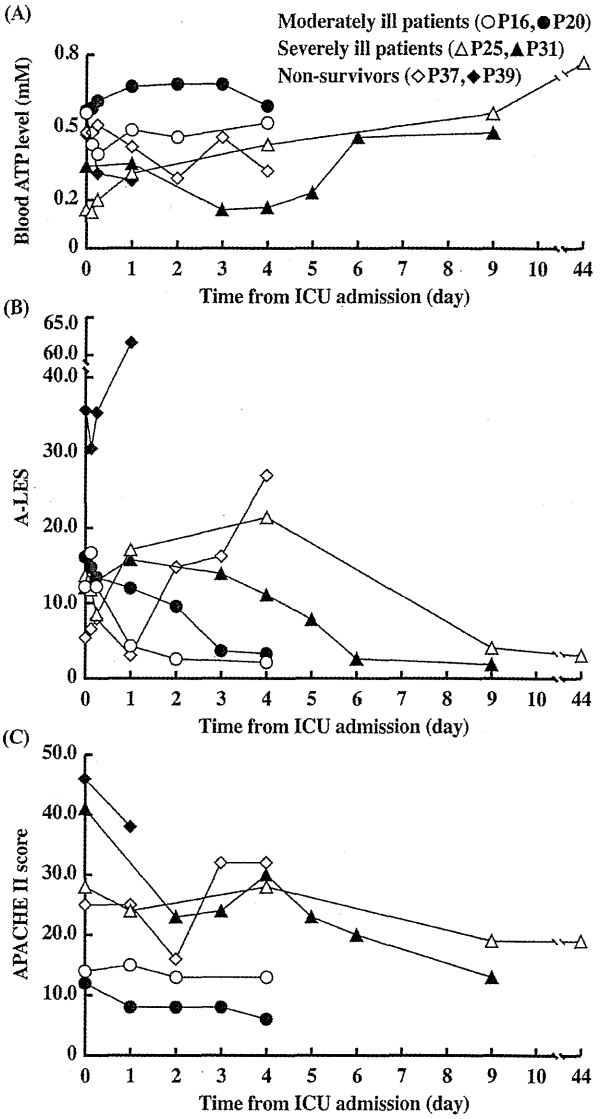

Figure 4. Changes in blood ATP level, A-LES, and APACHE II score of representative examples during the course of ICU stay. Serial changes in the levels of (A) blood ATP level, (B) A-LES, and (C) APACHE II score from admission to discharge from the ICU according to the severity of clinical condition (moderately and severely ill patients and non-survivors).
doi:10.1371/journal.pone.0060561.g004

at ICU discharge was 2.7 (2.1 to 3.3) and that of non-survivors was significantly high at 38.9 (21.0 to 67.9) ($P<0.01$) (Table 4). The A-LES was <20 in almost all moderately ill patients during ICU stay and was ≥20 in a large proportion of the non-survivors (Table 4 and Figure 3B). The results suggest that 20 is a critical cut-off value of A-LES for prediction of survival in the limited number of patients in this study. The change in A-LES ranged from 3.05 to 89.73 in non-survivors whereas the change in APACHE II score was ranged only from 17 to 46 (Table S3 and Figure 3B). These results suggest that A-LES provides better prognostic information compared to the APACHE II score. In addition to the value of A-LES during ICU admission, the AUC values for APACHE II, ATP and A-LES (Figure 3A) indicate that simple measurement of

blood ATP and A-LES at the time of ICU admission predicts mortality in a manner similar to APACHE II, the complex and time-consuming evaluation method.

Although ATP released from damaged cells and tissues [17,19] and from RBC in response to low PO_2, low pH and/or mechanical deformation [30,31] is an emergency signal alarmin, the levels in serum are difficult to monitor because the released ATP is rapidly degraded within few minutes [19]. The major source of ATP in the blood is the RBC and blood ATP levels change hourly with changes in energy and vital status of patients. Therefore, A-LES level is a real-time and sensitive biomarker of vital sign and a marker for prediction of mortality in critically ill ICU patients.

Conclusions

This is the first report on blood ATP levels and A-LES as an alarm biomarker for critical illness during ICU stay and for the prediction of outcome of clinically ill patients at the time of ICU admission, similar to APACHE II score. In addition, A-LES provided further evaluation score of illness severity during ICU stay in addition to APACHE II particularly for those critically ill patients with a score of ≥20.0.

Supporting Information

Table S1 Demographics and clinical details of moderately ill patients (APACHE II score<20). *Blood samples were collected at D0 = ICU day 0 (ICU admission), D1 = ICU day 1 (discharge or death from ICU within 24 hours) and D4 = ICU day 4 (discharge or death from ICU within 4 days). # Blood samples were collected from arterial blood (A) or central venous blood (CV). tHb = total hemoglobin; BS = blood sugar.
(DOC)

Table S2 Demographics and clinical details of severely ill patients (APACHE II score ≥20). *Blood samples were collected at D0 = ICU day 0 (ICU admission), D1 = ICU day 1 (discharge or death from ICU within 24 hours) and D4 = ICU day 4 (discharge or death from ICU within 4 days). # Blood samples were collected from arterial blood (A) or venous blood (V). For abbreviations, see Table S1.
(DOC)

Table S3 Demographics and clinical details of non-survivors. *Blood samples were collected at D0 = ICU day 0 (ICU admission), D1 = ICU day 1 (discharge or death from ICU within 24 hours) and D4 = ICU day 4 (discharge or death from ICU within 4 days). # Blood samples were collected from arterial blood (A). For abbreviations, see Table S1.
(DOC)

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Author Contributions

Conceived and designed the experiments: JC HK MN. Performed the experiments: RO MO EN KS MM KY. Analyzed the data: JC MH. Contributed reagents/materials/analysis tools: JC. Wrote the paper: JC HK.

References

1. Knaus WA, Draper EA, Wagner DP, Zimmerman JE (1985) APACHE II: a severity of disease classification system. Crit Care Med 13: 818–829.
2. Le Gall JR, Loirat P, Alperovitch A, Glaser P, Granthil C, et al. (1984) A simplified acute physiology score for ICU patients. Crit Care Med 12: 975–977.
3. Vincent JL, Moreno R, Takala J, Willatts S, De Mendonca A, et al. (1996) The SOFA (Sepsis-related Organ Failure Assessment) score to describe organ dysfunction/failure. On behalf of the Working Group on Sepsis-Related Problems of the European Society of Intensive Care Medicine. Intensive Care Med 22: 707–710.
4. Teasdale G, Jennett B (1974) Assessment of coma and impaired consciousness. A practical scale. Lancet 2: 81–84.
5. Holtfreter B, Bandt C, Kuhn SO, Grunwald U, Lehmann C, et al. (2006) Serum osmolality and outcome in intensive care unit patients. Acta Anaesthesiol Scand 50: 970–977.
6. Schonhöfer B, Lefering R, Suchi S, Köhler D (2002) Survey of evaluation of scoring systems by intensive care physicians. Intensivmed 39: 240–245.
7. Ishizaka A, Tono-Oka T, Matsumoto S (1984) Evaluation of the proliferative response of lymphocytes by measurement of intracellular ATP. J Immunol Methods 72: 127–132.
8. Garewal HS, Ahmann FR, Schifman RB, Celniker A (1986) ATP assay: ability to distinguish cytostatic from cytocidal anticancer drug effects. J Natl Cancer Inst 77: 1039–1045.
9. Sevin BU, Peng ZL, Perras JP, Ganjei P, Penalver M, et al. (1988) Application of an ATP-bioluminescence assay in human tumor chemosensitivity testing. Gynecol Oncol 31: 191–204.
10. Crouch SP, Kozlowski R, Slater KJ, Fletcher J (1993) The use of ATP bioluminescence as a measure of cell proliferation and cytotoxicity. J Immunol Methods 160: 81–88.
11. Garland JM, Halestrap A (1997) Energy metabolism during apoptosis. Bcl-2 promotes survival in hematopoietic cells induced to apoptosis by growth factor withdrawal by stabilizing a form of metabolic arrest. J Biol Chem 272: 4680–4688.
12. Nakamura N, Wada Y (2000) Properties of DNA fragmentation activity generated by ATP depletion. Cell Death Differ 7: 477–484.
13. Cook SP, McCleskey EW (2000) ATP, pain and a full bladder. *Nature*; 407: 951–952.
14. Törnroth-Horsefield S, Neutze R (2008) Opening and closing the metabolite gate. Proc Natl Acad Sci U.S.A. 105: 19565–19566.
15. Manson J, Thiemermann C, Brohi K (2012). Trauma alarmins as activators of damage-induced inflammation. Br J Surg 99: 12–20.
16. Ellsworth ML, Ellis CG, Goldman D, Stephenson AH, Dietrich HH, et al. (2009) Erythrocytes: oxygen sensors and modulators of vascular tone. Physiology 24: 107–116.
17. Oppenheim JJ, Yang D (2005) Alarmins: chemotactic activators of immune responses. Curr Opin Immunol 17: 359–365.
18. Lotze MT, Zeh HJ, Rubartelli A, Sparvero LJ, Amoscato AA, et al. (2007) The grateful death: damage-associated molecular pattern molecules and reduction/oxidation regulate immunity. Immunol Rev 220: 60–81.
19. Kobayashi T, Kouzaki H, Kita H (2010) Human eosinophils recognize endogenous danger signal crystalline uric acid and produce proinflammatory cytokines mediated by autocrine ATP. J Immunol 184: 6350–6358.
20. Robinson K, Kongable GL (2010) Lactate in critical illness-implications for monitoring. US Respiratory Disease 6: 52–55.
21. Fredriksson K, Hammarqvist F, Strigård K, Hultenby K, Ljungqvist O, et al. (2006) Derangements in mitochondrial metabolism in intercostal and leg muscle of critically ill patients with sepsis-induced multiple organ failure. Am J Physiol Emdocrinol Metab 291: E1044–E1050.
22. Brealey D, Brand M, Hargreaves I, Heales S, Land J, et al. (2002) Association between mitochondrial dysfunction and severity and outcome of septic shock. Lancet 360: 219–223.
23. Carré JE, Orban JC, Re L, Felsmann K, Iffert W, et al. (2010) Survival in critical illness is associated with early activation of mitochondrial biogenesis. Am J Respir Crit Care Med 182: 745–751.
24. Pan HY, Yamada H, Chida J, Wang S, Yano M, et al. (2011) Up-regulation of ectopic trypsins in the myocardium by influenza A virus infection triggers acute myocarditis. Cardiovasc Res 89: 604–613.
25. Kubota M, Chida J, Hoshino H, Ozawa H, Koide A, et al. (2012) Thermolabile CPT II variants and low blood ATP levels are closely related to severity of acute encephalopathy in Japanese children. Brain Dev 34: 20–27.
26. Chida J, Yamane K, Takei T, Kido H (2012) An efficient extraction method for quantitation of adenosine triphosphate in mammalian tissues and cells. Anal Chim Acta 727: 8–12.
27. Knaus WA, Draper EA, Wagner DP, Zimmerman JE (1986) An evaluation of outcome from intensive care in major medical centers. Ann Intern Med 104: 410–418.
28. Weil MH, Michaels S, Rackow EC (1987) Comparison of blood lactate concentrations in central venous, pulmonary artery, and arterial blood. Crit Care Med 15: 489–490.
29. Schenten D, Medzhitov R (2011) The control of adaptive immune responses by the innate immune system. Adv Immunol 109: 87–124.

30. Sprague RS, Ellsworth ML, Stephenson AH, Lonigro AJ (1996) ATP: the red blood cell link to NO and local control of the pulmonary circulation. Am J Physiol 271: H2717–H2722.

31. Ellsworth ML, Ellis CG, Goldman D, Stephenson AH, Dietrich HH, et al. (2009) Erythrocytes: oxygen sensors and modulators of vascular tone. Physiology 24: 107–116.