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Lactosyl derivatives function in a rat model of severe burn shock by acting as antagonists against CD11b of integrin on leukocytes

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Abstract Severe burn shock remains an unsolved clinical problem with urgent needs to explore novel therapeutic approaches. In this study, the *in vivo* bioactivity of a series of synthetic lactosyl derivatives (oligosaccharides) was assessed on rats with burn shock to elucidate the underlying mechanisms. Administration of An-2 and Gu-4, two lactosyl derivatives with di- and tetravalent β-D-galactopyranosyl-(1-4)-β-D-glucopyranosyl ligands, significantly prolonged the survival time ($P<0.05$ *vs*. saline), stabilized blood pressure and ameliorated the injuries to vital organs after burn. Flow chamber assay displayed that An-2 and Gu-4 markedly decreased the adhesion of leukocytes to microvessel endothelial cells. Competitive binding assay showed that a CD11b antibody significantly interrupted the interaction of An-2 and Gu-4 with leukocytes from rats with burn shock. With fluorescent microscopy, we further found that the oligosaccharides were selectively bound to leukocytes and with a colocalization of CD11b on the cell membrane. Interestingly, the lectin domain-deficient form of CD11b failed to bind with An-2 and Gu-4. The results suggest that both An-2 and Gu-4 significantly inhibit the adhesion of leukocytes to endothelial cells by binding to CD11b and thereby exert protective effects on severe burn shock.

Keywords Leukocyte–endothelial cell interaction · Adhesion molecule · Oligosaccharide · Integrin · Burn shock

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Shock is a clinical syndrome associated with inadequate blood flow to vital organs and tissues causing life-threatening cellular dysfunction [1]. During shock, the widespread activation of leukocytes, lymphocytes and endothelial cells by an array of inflammatory mediators (including cytokines, chemokines, prostaglandins and lipid mediators, and reactive oxygen species) induces upregulation of adhesion molecules, promoting adhesion and extravasation of neutrophils and monocytes through capillaries [2]. The damage caused by intra- and extravascular phagocytic cells together with injuries from other pathways ultimately leads to the development of multiple-organ failure, in which the lung, liver, heart and kidney are the major vital organs involved [3].

Previous studies demonstrated that the interaction between leukocytes and endothelial cells plays an important

role in the pathogenesis of shock [4, 5]. It was shown *in vivo* that leukocyte adhesion to the blood vessel walls and obstructing capillary flow was the major mechanism for no-reflow and inadequate blood perfusion of tissues during shock [6]. Leukocyte adhesion to endothelial cells is a tightly coordinated multistep process mediated by adhesion molecules [4, 7]. This knowledge facilitated our understanding of its involvement in ischemia–reperfusion injury (IRI), burns and thermal injuries, inflammation and cancer metastasis [4, 7, 8]. Preclinical and clinical trails to treat diseases mediated by unwanted leukocyte adhesion employ molecules that interfere with the adhesion of leukocytes to endothelial cells [9, 10]. Many monoclonal antibodies (mAbs) against adhesion molecules have been tested on various disease models; some showed encouraging results [11, 12]. Soluble ligands, such as P-selectin glycoprotein ligand-1 (PSGL-1), a common ligand for selectins, were investigated in traumatic shock and inflammation models [13]. Despite their effectiveness in animals, the clinical application of these strategies was limited by the shortcomings and/or side effects. For example, therapeutic antibodies, as large exogenous immunogenic proteins, may induce antigenicity [14].

To overcome this problem, low molecular weight inhibitors like oligosaccharides can be pursued [15]. Some glycosyl moieties on adhesion molecules or their ligands were directly involved in the interactions between adhesion molecules expressed on the surface of leukocytes and endothelial cells. Interestingly, a small tetrasaccharide, sialyl Lewis X (sLeX), a capping component of selectin ligands, conjugated to all selectins [16, 17]. This lead to the exploration of effective selectin inhibitors based on the structure of sLeX. A variety of selectin inhibitors were discovered, including sLeX-containing oligosaccharides, modified LeX and other synthetic compounds [15]. Studies on soluble lectins, also known as galectins, were also pursued in search of novel anti-adhesion drugs. Galectins represent a growing multifunctional superfamily and some of them were found to participate in the adhesion of neutrophils, eosinophils and macrophages through interaction with their ligands, such as CD11b, in a lactose dependent manner [18]. Lactosamine, the basic repeat unit within the backbone of the glycoconjugates, also attracted much interest [19]. Lactose and its derivatives were used in anti-adhesion assays as well as anti-tumor metastasis studies [20, 21]. Recently, the acidic fraction of lactose-derived oligosaccharides from human milk was reported to potently inhibit leukocyte adhesion [22].

Based on the findings above, we hypothesized that lactosyl derivatives may function as inhibitors against leukocyte adhesion and may contribute to the treatment of severe burn shock. In this article, we report the therapeutic effects of two synthesized lactose-derived oligosaccharides, An-2 and Gu-4, on a burn shock model of rats. Investigations of the underlying mechanisms showed that these two bioactive saccharides exerted their biological effects by targeting CD11b, the β2 integrin subunit, on the surface of leukocytes.

Materials and methods

Synthesis of lactosyl derivatives

The lactosyl derivatives were designed and synthesized by attaching various numbers (n=2, 4) of oligosaccharides to different linkers following the procedures by Li *et al*. [23, 24]. In this study, oligosaccharides are referred to β-D-galactopyranosyl-(1-4)-β-D-glucopyranosyl, while linkers are represented by 2-amino glycerol or glutamic acid or polyethylene glycol (PEG) (Fig. 1). All compounds were prepared, and their structures were confirmed by nuclear magnetic resonance (NMR), mass-spectrometry (MS) and elemental analysis.

Recombinant DNA constructs

The plasmids expressing human CD1b and CD18 were gifts from Professor Timothy Springer (Harvard Medical School, Harvard University, USA). The DNA coding sequences of CD11b and CD18 were amplified by PCR with primers specific for CD11b (Forward: 5′-GAAGAA GCTTGCCACCATGGCTCTCAGAGTCCTTCTG-3′; Reverse: 5′-ATTATCTAGA CTACTGGGGTTCGG CCCCCG-3′) and CD18 (Forward: 5′-TAATAAGCTTGC CACCATGCTGGGCCTGCGC-3′; Reverse: 5′-CGG CTCTAGACTAACTCTCAGCAAACTTGG-3′), and subcloned into pcDNA3 vector with enzyme sites of *Hin*d III and *Xba* I. The constructs were confirmed by DNA sequencing, and named as pcDNA3/CD11b and pcDNA3/CD18 respectively. The construct expressing the C-terminal lectin domain-deficient form of CD11b (pcDNA3/CD11bΔL) was generated by PCR with primers 5′-ACGGAGACCAAAGTGGAGCCGTTCG-3′ (sense primer) and 5′-TACCAGTCCATCCATTGTGAGGTCCTGG-3′ (anti-sense primer) and confirmed by DNA sequencing.

Production of severe burn shock model in rats

Specific pathogen free (SPF) Sprague–Dawley rats were purchased from the Experimental Animal Center of Southern Medical University in Guangzhou, China. The experimental protocols to produce severe burn shock model were reviewed and approved by the Animal Care Committee of Southern Medical University and performed in accordance with the guidelines of the National Institute of Health

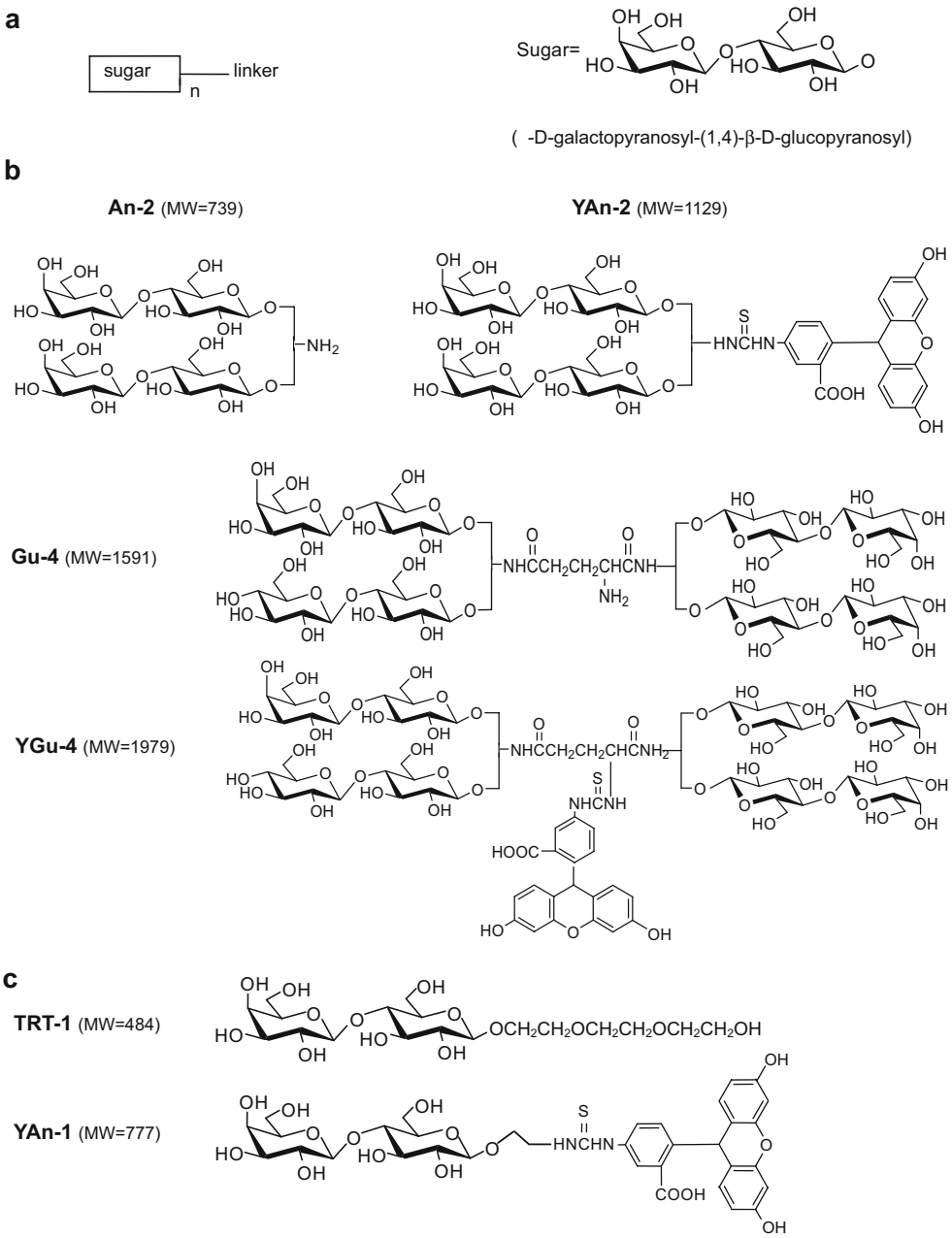

Fig 1 Chemical structures of the lactosyl derivatives. **a** General formula of the synthesized oligosaccharides and the molecular formula of the sugar that is attached to the linker as a basic component. Here, *n* indicates the number of attached sugars; 'linker' is referred to either as 2-amino glycerol or glutamic acid or PEG. **b** The molecular formulas of An-2 and Gu-4, two oligosaccharides exhibiting a significant anti-shock effect, and the molecular formulas of FITC labeled An-2 and Gu-4, named YAn-2 and YGu-4, are shown. **c** The molecular formulas of TRT-1 and YAn-1, synthetic sugars used as control. The molecular weights (*MW*) are indicated

of the USA. In brief, 6–8 week old male rats weighing 200–220 g were first anesthetized with 13.3% urethane and 0.5% chloralose (0.6 ml/100 g, i.p.). The right carotid artery and the left jugular vein were cannulated to continuously monitor blood pressure with a pressure transducer (Statham P23XL, Germany) and infusion of solutions with or without drugs, respectively. No extra fluid resuscitation was performed to the burned rats. The mean arterial blood pressure (MAP) was calculated by adding one third of the differential pressure (systolic minus diastolic) to diastolic pressure. The lower trunk and lower extremities (35–40% total body surface) of rats were scalded with 80°C water for 30 s to produce the burn shock; the onset of shock was confirmed by recording a sustaining decline of MAP in 3 h. Our and others' previous studies have shown that such a treatment serves to develop a severe shock state that mimics the condition of patients with severe burn with persistent low perfusion; and leukocyte adhesion on venular walls plays a pivotal role in the development of low blood pressure and organ damage of burn shock [5, 25–27]. So, this model is suitable to evaluate the effectiveness of the synthetic oligosaccharides.

In vivo pharmacokinetic analysis of the lactosyl derivatives

To determine the pharmacokinetics of the lactosyl derivatives, fluorescein isothiocyanate (FITC)-labeled An-2 and

Gu-4 were administrated separately to 6–8 week old normal male rats weighing 200–220 g by intravenous injection (20 nmol/100 g body weight in 0.4 ml saline). A series of blood samples (0.3 ml each) were collected at different time points through the carotid artery catheter and equal volume of saline was reinfused to maintain blood volume. Serum was separated from blood samples by centrifugation at 1,200 rpm for 5 min and stored at −20°C for later analysis of fluorescence intensity. For analysis, a 100 μl aliquot of each sample was placed in a separate well of a 96-well plate. The fluorescence intensities of the samples were then read on a microplate reader (SpectraMax M5, Molecular Devices Corporation, USA) at an excitation wavelength of 485 nm. Urine samples from rats were collected before or at a time interval of 1 h after administration and subjected to the same procedure described above for detection of fluorescence intensity.

Effect of the lactosyl derivatives on the survival time in burn shock

To test the biological effect of the lactosyl derivatives on rats with burn shock, 80 male rats were randomly assigned to equal groups (n=8). Different amounts of lactosyl derivatives (5, 20 and 80 nmol/100 g body weight in 0.4 ml saline, respectively) were administrated by intravenous injection 30 min after scalding to determine the dose–effect relationship of the lactosyl derivatives. The survival time of each rat was recorded. Equal volume saline and a synthetic sugar, TRT-1, were used as controls.

According the pharmacokinetics of the lactosyl derivatives, most of the An-2 and Gu-4 in the blood was discharged through the kidney within 1 h. To see if repeated doses of An-2 or Gu-4 achieve a better therapeutic effect on burned rats, another 40 male rats were randomly assigned to five groups (n=8). The oligosaccharides (20 nmol/100 g body weight in 0.4 ml saline) were administrated intravenously to the burned rats in single dose or four times at a time interval of 1 h between administrations. As described above, an equal volume of saline was used as control.

Histological examination of vital organs

To test the therapeutic effect of the lactosyl derivatives on the tissues of vital organs after burn, 32 male rats were randomly divided into four equal groups and subjected to the same operation procedures as delineated above. Ten hours after scalding, lung, kidney, heart and liver were collected for histology. Tissue samples were fixed in 10% formaldehyde solution. Paraffin-embedded tissue sections were stained with hematoxylin and eosin (H&E) and observed under an optical microscope (DM-RA2, Leica) [28].

Cell culture and DNA transfection

Rat lung microvascular endothelial cells (RLMVECs) were isolated and grown with a method described previously [29]. In brief, pathogen-free male Sprague–Dawley rats (4–5 week old) weighing 70–100 g were anesthetized and exsanguinated. The lung tissue was washed three times with Dulbecco's Modified Eagle's Medium (DMEM) containing antibiotics and 15 mmol/l hydroxyethyl piperazine ethanesulfonic acid (HEPES, HyClone). The outer 3–5 mm peripheral lung tissues were dissected from lung lobes, washed with DMEM and finely minced using iris scissors. The minced lung tissues were seeded on a culture plate coated with gelatin (Sigma-Aldrich) and supplemented with DMEM containing 20% fetal bovine serum (HyClone). The plates were kept in a humidified incubator at 37°C with 5% CO_2 for 4 days. The tissue debris was then removed by washing with culture medium and the remaining adherent cells were cultured until a cell monolayer was formed. The cells were passed by digestion with 0.25% trypsin to Petri dishes or glass slides in culture plate and allowed to grow to confluence for further experiments. Endothelial cells were initially identified by their cobblestone morphology and growth pattern and further confirmed by immunohistochemical staining with a specific antibody against factor VIII-related antigen (Santa Cruz Biotechnology) [30, 31].

Murine fibroblast NIH3T3 cells were purchased from American Type Culture Collection (ATCC) and were grown in DMEM with 10% fetal bovine serum (Hyclone) and 0.1 mmol/l non-essential amino acids at 37°C with 5% CO_2. Transient transfection was performed by using Lipofectamine (Invitrogen) in NIH3T3 cells with pcDNA3/CD11b, pcDNA3/CD11b plus pcDNA3/CD18, pcDNA3/CD11bΔL, pcDNA3/CD11bΔL plus pcDNA3/CD18 or equal amount of pcDNA3 blank vector, respectively.

Preparation of whole blood leukocytes

Blood was withdrawn for isolation of leukocytes from rats without or with burn shock 3 h after scalding. The heparinized whole blood was mixed with red blood cell lysis buffer ($KHCO_3$ 1 g, NH_4Cl 8.3 g, EDTA–Na_2 37 mg, add sterile water to 1,000 ml, adjust pH to 7.2 with HCl) at a ratio of 1:10 (v/v). The mixture was incubated at room temperature for 5 min with intermittent shaking. The reaction was stopped by dilution of the mixture with 20 volumes of isotonic phosphate buffered saline (PBS). After centrifugation at 400×g for 5 min, the supernatant was removed and the cell pellet was resuspended in PBS. The cells were adjusted to a concentration of 1×10^6 cells/ml for further experiments.

Leukocyte–endothelium adhesion assay

A parallel-plate flow chamber previously described by Hochmuth *et al*. [32] and Gallik *et al*. [33] was used to examine the adhesion of leukocytes to endothelium. The assembled chamber mounted with a glass slide seeded with RLMVECs was placed on an inverted microscope (IX71, Olympus) stage and attached to a perfusion pump (model 44, Harvard Apparatus) to maintain 37°C. A suspension of leukocytes (1×10^6/ml) treated with saline, monoclonal antibody (mAb) for CD62L (20 ng/ml) (#304802, Biolegend), An-2 (4 nmol/ml), Gu-4 (4 nmol/ml) or TRT-1 (4 nmol/ml) for 10 min respectively, was infused through the perfusion system and incubated with RLMVECs in the flow chamber for 20 min.

Before perfusion, leukocytes in ten random microscopic fields were counted and the average was taken as the control count for calculation of leukocyte adhesion. Then the flow chamber was perfused with Hank's balanced salt solution (HBSS) at a shear stress in the range of 0.125–5.621 dynes/cm^2. The perfusion at each shear stress was maintained for 2 min and followed by temporary stasis for quantitation of adherent leukocytes in ten random microscopic fields. The adhesion rate (percent) under a definite shear stress was obtained from the ratio of average adherent leukocyte count and the control count [33]. The images were captured by a CCD camera (Panasonic, Japan) and used for calculation of the adhesion rate.

Fluorescence microscopic observation

RLMVECs, NIH3T3 cells and leukocytes isolated from rats were used to detect the binding activity of the lactosyl derivatives, An-2 and Gu-4. RLMVECs and NIH3T3 cells were cultured for 24 h before the binding assay. Leukocytes in suspension were added to Petri dishes and incubated at 37°C, 5% CO_2 for 1 h, permitting cells to adhere to the bottom surface. FITC-labeled lactosyl derivatives were diluted to 20 pmol/μl with isotonic PBS and added to the Petri dishes (100 μl/dish). The dishes were kept at room temperature for 10 min and then washed with PBS for three times to remove the un-conjugated sugars. The images of the cells bound with An-2 or Gu-4 were observed under a fluorescence microscope (DM-RA2, Leica).

After transfection with the plasmids expressing full length or lectin domain-deficient form of CD11b for 24 h, NIH3T3 cells were subjected to immunofluorescent staining with Phycoerythrin (PE)-conjugated monoclonal antibody against human CD11b (sc-1186, Santa Cruz) and FITC-labeled lactosyl derivatives, to clarify whether the interaction of CD11b with An-2 and Gu-4 is dependent on its C-terminal lectin domain. The detailed procedure for the fluorescent microscopy was as described above.

Flow cytometric analysis

Flow cytometry was performed on a FACScan flow cytometer (Beckman Coulter, USA) for determination of conjugation kinetics of An-2 and Gu-4 with leukocytes and quantitation of CD62L and CD11b expressed on the surface of leukocytes. Leukocytes were isolated from whole blood aliquots of rats as described above. Cells were stained with a FITC-labeled lactosyl derivative, YAn-2 or YGu-4 (2 nmol/μl), Phycoerythrin (PE)-conjugated CD62L mAb (#sc-13505, Santa Cruz Biotechnology) or CD11b mAb (#GTX76061, Gene Tex), respectively, in a 100 μl system at 4°C in the dark for 10 min. Analysis was performed on 10,000 cells using an argon laser beam (488 nm) and a helium/neon laser beam (594 nm). Different cell subsets were characterized by forward and side light scattering. Mean logarithmic fluorescence intensity (MLFI) was chosen as the measurement of the brightness of stained cells. Non-specific background fluorescence was evaluated by the fluorescence associated with the detected cells upon incubation with antibodies with crossmatched isotypes.

To test whether CD11b mAb could disrupt the coupling of An-2 or Gu-4 with leukocytes, a competitive binding assay was carried out separately with a preselected amount of a FITC-labeled lactosyl derivative and different amounts of the PE-labeled CD11b mAb or a PE-conjugated mouse IgG2a isotype control antibody (#IC003P, R&D Systems, Inc.) with final concentrations ranging between 0 and 50 ng/μl. After centrifugation at $400\times g$ for 5 min, cells were washed three times with ice-cold PBS and resuspended in 1 ml PBS for flow cytometric analysis.

Confocal microscopy examination

To determine whether the lactosyl derivatives, An-2 and Gu-4 colocalized with CD11b on the cell membrane, leukocytes from rats without or with burn shock were isolated as described above for confocal microscopy. After three times wash with ice-cold PBS following centrifugation at $400\times g$ for 5 min, cells were resuspended with 1 ml of PBS. Cell suspension was added to culture dishes to allow adhesion of leukocytes. The non-adhered cells were aspirated and adherent ones were stained with a PE-conjugated CD11b mAb (#GTX76061, Gene Tex) and YGu-4 or YAn-2 (2 nmol/μl) simultaneously at 4°C in the dark for 10 min. Conjugation patterns of CD11b mAb and YGu-4 or YAn-2 on leukocytes were examined with a laser confocal scanning microscope (Leica TCS SP2, Leica Microsystems Heidelberg, Germany). NIH3T3 cells subjected to the same staining procedure served as negative controls.

Statistical analysis

All data are presented as mean ± standard deviation (SD). The data were analyzed with the Statistical Package for Social Sciences (Version 11.0). One-way ANOVA was used to evaluate the significance of inter-group differences. Survival of rats was analyzed with Kaplan–Meier survival analysis with the log-rank test for between-group comparisons. $P<0.05$ was considered statistically significant.

Results

Structures of the synthetic lactosyl derivatives

In this study, oligosaccharides were designed and chemically synthesized following a general formula of sugar and linker composition (Fig. 1a). All the synthetic oligosaccharides were tested on a burn shock model of rats, and oligosaccharides designated as An-2 and Gu-4, significantly prolonged the survival time and slowed down the rapid decline of MAP after burn, while the others (such as TRT-1) failed to achieve a significant effect. To further elucidate the underlying biological mechanism, these two sugars were labeled with FITC and named YAn-2 and YGu-4, respectively. The structures of An-2, Gu-4 and their FITC-labeled derivatives are illustrated in Fig. 1b. The structures of the control oligosaccharides, TRT-1 and YAn-1 are illustrated in Fig. 1c.

Pharmacodynamics of the lactosyl derivatives

In order to determine the pharmacodynamics of the oligosaccharides, FITC-labeled An-2 and Gu-4 were intravenously injected into rats for measurement of fluorescent intensity in blood and urine samples. We found that the fluorescent intensity of the serum declined quickly after administration of the FITC-labeled oligosaccharides with a half-life of about 30 min (Fig. 2a). Simultaneously, following the decline of the serum fluorescence intensity, the fluorescence intensity of urine increased significantly 1 h after the administration of the synthesized oligosaccharides (Fig. 2b), indicating that the oligosaccharides in the blood were quickly cleared through the kidney. This experiment was repeated three times and comparable results were obtained.

Effects of An-2 and Gu-4 on the survival of rats with burn shock

Without therapeutic intervention, the burned rats invariantly die 6–15 h after the injury. As shown in Fig. 3, application of An-2 and Gu-4 significantly prolonged the survival time of the burned rats in a dose-dependent manner. Administration of intermediate doses of An-2 or Gu-4 (20 nmol/100 g body weight) served to increase the mean survival time of the rats with burn shock to 21.5±2.20 and 24.0±3.96 h, respectively; high dose of the oligosaccharides (80 nmol/100 g body weight) showed even a better therapeutic effect. The mean survival times in the high dose groups were 38.12±2.99 and 47.80±6.39 h, respectively, which were significantly longer than that of the saline treated group (10.43±2.95 h) and the TRT-1 control group (11.36±4.22 h) ($P<0.05$) (Fig. 3a).

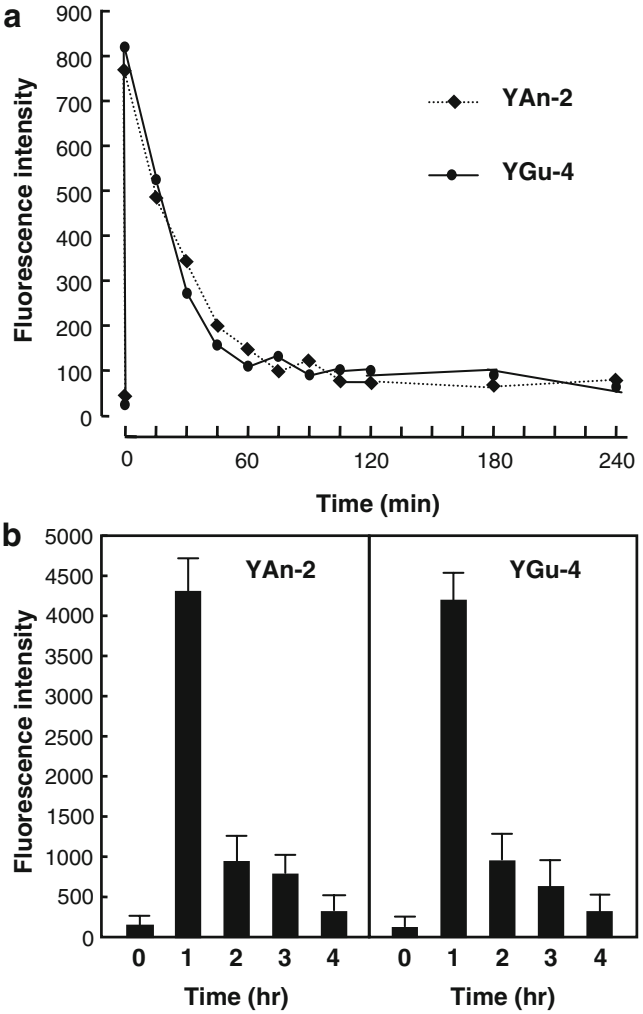

Fig 2 Pharmacokinetic analysis of An-2 and Gu-4 in rats. **a** Dynamic measurement of fluorescence intensity of serum from rats subjected to intravenous injection of YAn-2 or YGu-4. **b** The fluorescence intensity of urine from rats. Urine samples were collected from rats at a time interval of 1 h after intravenously administration of YAn-2 or YGu-4. Data shown here represent three individual rats ($n=3$)

Pharmacokinetic analysis revealed that both An-2 and Gu-4 were quickly cleared with a serum concentration close to the basal value within 1 h (Fig. 2). Therefore, we treated rats with repeated doses (20 nmol/100 g body weight, 4 times) of the lactosyl derivatives once per hour. Kaplan–Meier survival curve analysis demonstrated that An-2 and Gu-4 had a significant effect on the survival of rats with burn shock

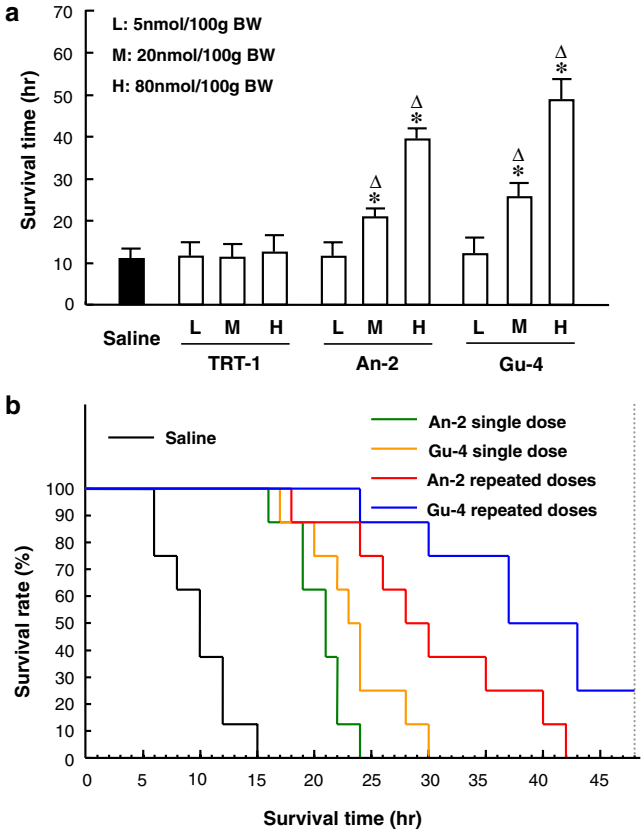

Fig 3 Effects of An-2 and Gu-4 on the survival of rats with severe burn shock. **a** Survival time of rats with burn shock on the treatment of the oligosaccharides. Data was shown as mean ± SD (n=8). P< 0.05 *vs*. saline group (*) or sugar control group (Δ); **b** Kaplan–Meier survival analysis of burn shock rats treated with saline (*black line*), or single dose (20 nmol/100 g BW) of An-2 (*green line*) or Gu-4 (*yellow line*), or repeated doses (20 nmol/100 g BW for four times with a time interval of 1 h) of An-2 (*red line*) or Gu-4 (*blue line*). Administration of An-2 or Gu-4 significantly prolonged the survival time of rats with burn shock (P<0.05 *vs*. saline group). Repeated doses of the oligosaccharides achieved a better therapeutic effect than single dose treatment on the burned rats (P<0.05 *vs*. single dose group). *BW* Body weight

(P<0.05) (Fig. 3b). We found that repeated dosing of An-2 or Gu-4 significantly prolonged the survival time of rats with burn shock from 12.56±3.57 h of the control to 30.09±2.11 and 40.11±3.08 h, respectively, with a much better outcome than the single dose group (Fig. 3b, P<0.05).

Effects of An-2 and Gu-4 on mean arterial pressure of rats with burn shock

Along with the observation of the survival of rats with burn shock, we also checked whether the oligosaccharides affected the mean arterial pressure (MAP) of burned rats. We found that administration of An-2 or Gu-4, but not TRT-1, significantly offset the decrease in blood pressure of rats with burn shock (P<0.05) (Fig. 4a). The MAP of rats with burn shock declined dramatically 3 h after treatment with saline, while the treatment with An-2 or Gu-4 significantly alleviated the decrease of blood pressure of the burned rats (Fig. 4b). Although differences existed among individual rats in each group, the overall pattern of the blood pressure course was similar.

Protective effects of An-2 and Gu-4 on major organs of burned rats

With the experimental results above, we hypothesized that An-2 or Gu-4 protects rats from organ damage that may be caused by insufficient blood perfusion after burn. Histopathological examination showed that there was significant congestion of blood and infiltration of polymorphonuclear leukocytes into all examined major organs after burn shock (10 h after treatment) compared with normal control rats (Fig. 5). Additional pathological changes, including septal thickening in the lung, hydropic degeneration of the proximal convoluted tubule and loss of Bowman's space in

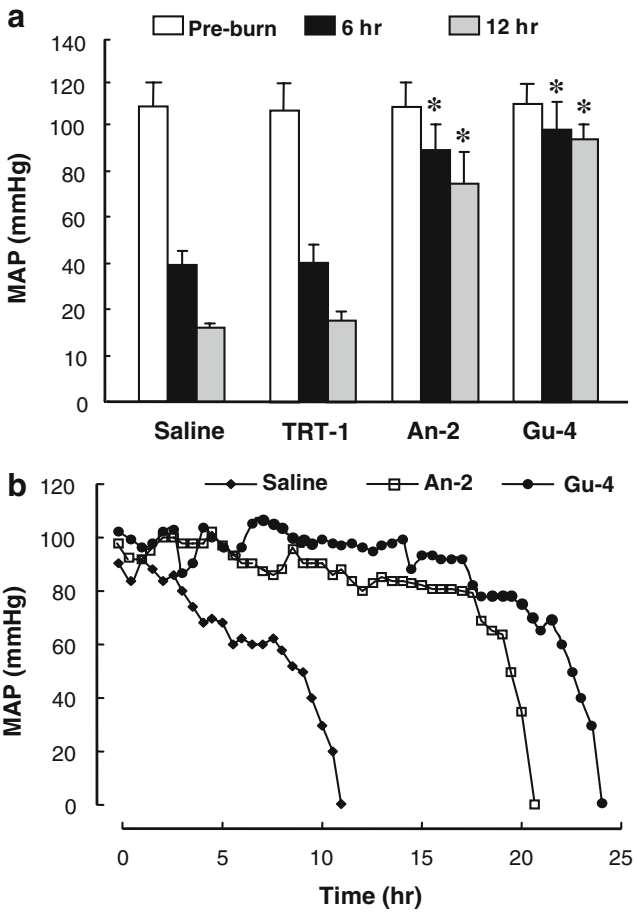

Fig 4 Effects of An-2 and Gu-4 on mean arterial pressure of rats with burn shock. **a** Mean arterial pressure (*MAP*) of rats with severe burn shock after treatment with saline or with the oligosaccharides for 6 or 12 h. Data was shown as mean ± SD (n=8). **b** Representative (one rat in each group) dynamic changes of MAP of burn shock rats treated with single dose of saline or the oligosaccharides

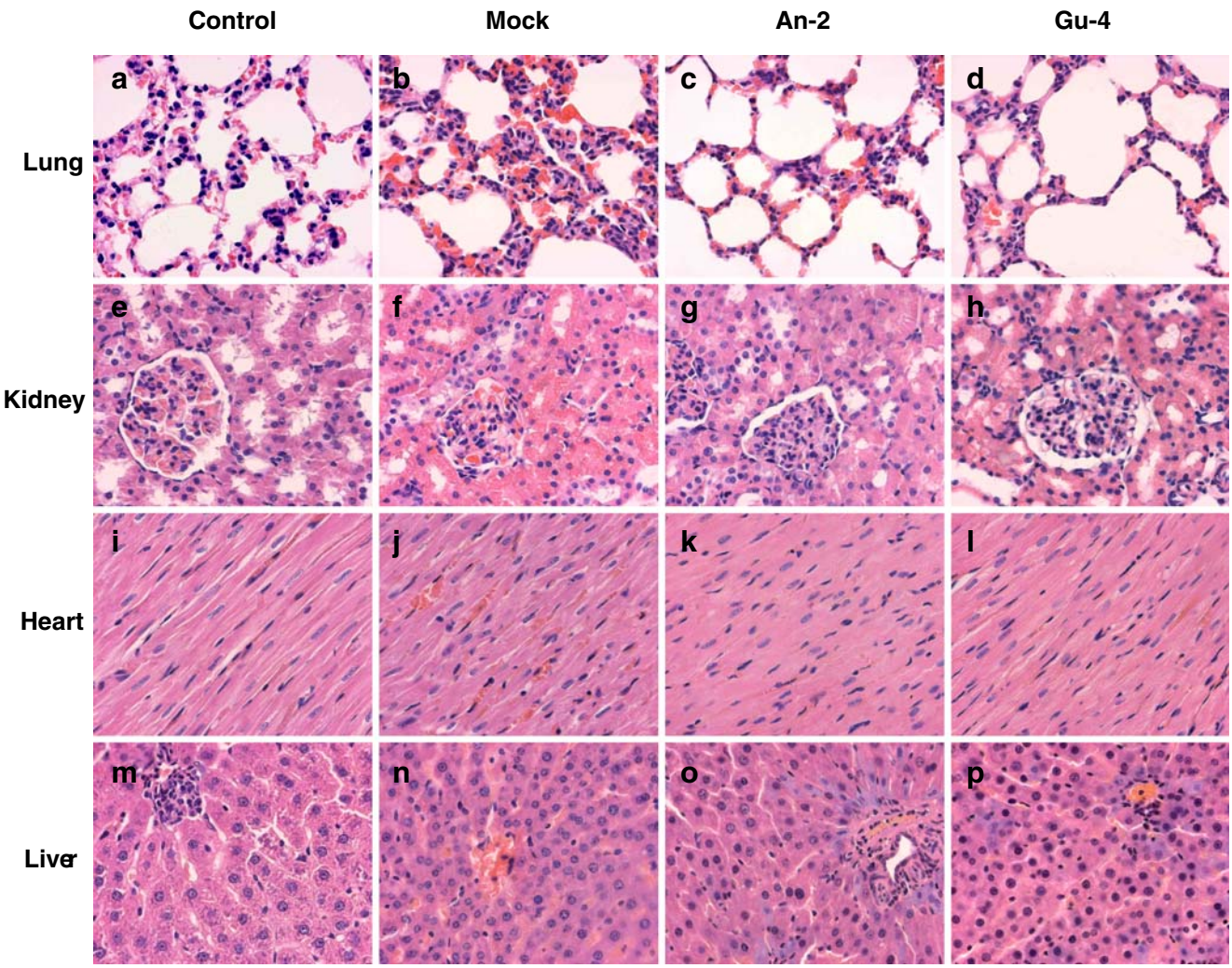

Fig 5 An-2 and Gu-4 alleviate histological damages of vital organs of rats with burn shock. Hematoxylin and eosin (H&E) staining of tissue sections of lung (**a**–**d**), kidney (**e**–**h**), heart (**i**–**l**) and liver (**m**–**p**) from normal rats (**a**, **e**, **i** and **m**) or burn shock rats treated with saline (**b**, **f**, **j** and **n**), An-2 (**c**, **g**, **k** and **o**) or Gu-4 (**d**, **h**, **l** and **p**), respectively. Original magnification, ×400

the kidney, congestion of coronary capillaries and degeneration of adipose tissue in the liver were also visible in the rats with burn shock. In contrast, in all vital organs from burned rats treated with An-2 and Gu-4, blood congestion and leukocyte infiltration were significantly alleviated, and other tissue damage was also attenuated (Fig. 5). Furthermore, we also saw that Gu-4 more potently suppressed pathological changes in rats with burn shock than did An-2.

Both An-2 and Gu-4 blocked the adhesion of leukocytes to RLMVECs

To test whether the lactose-derived oligosaccharides, An-2 and Gu-4, were involved in the adhesion between leukocytes and endothelial cells, a flow chamber assay was performed. The results showed that treatment with An-2 or Gu-4 significantly decreased the adhesion rate of leukocytes to RLMVECs on shear stresses in the range between 0.311 and 1.251 dynes/cm^2, indicating that they potently inhibited leukocyte–endothelial cell adhesion. As a positive control, the administration of anti-CD62L mAb to inhibit L-selectin -mediated leukocyte attachments to endothelium significantly decreased the adhesion of leukocytes to the endothelial cell monolayer ($P<0.05$), while the negative control, TRT-1, failed to show any inhibition effect. Notably, at higher levels of shear stress Gu-4 more potently inhibited adhesion between leukocytes and endothelial cells ($P<0.05$) (Fig. 6), correlating with its stronger protective effects in vital organs.

Leukocytes as target cells for An-2 and Gu-4

To establish the anti-adhesive effect of An-2 and Gu-4, we sought to identify the target cells of these two

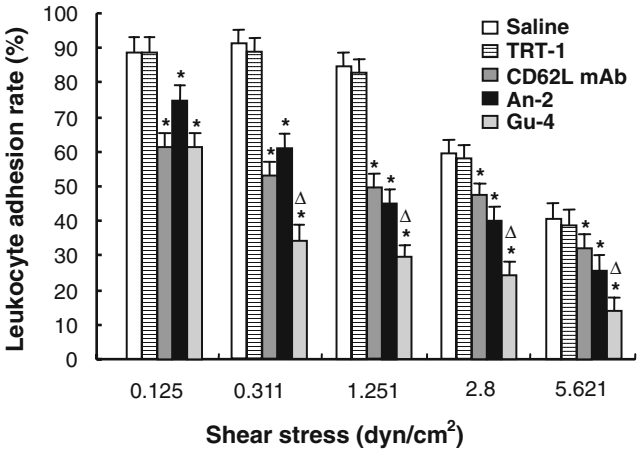

Fig 6 An-2 and Gu-4 inhibit the adhesion of leukocytes from burn shock rats with RLMVECs. Leukocytes from severe burn shock rats were first treated with saline, TRT-1, CD62L mAb, An-2 or Gu-4 for 10 min, respectively, and then subjected to a flow chamber assay. Adhesion rate of leukocytes to RLMVECs at different shear stresses were calculated and shown as mean ± SD. *$P<0.05$ *vs*. saline; $\Delta P<0.05$ *vs*. CD62L mAb

oligosaccharides. It is well established that adhesion molecules on the cell surface mediate the interaction between leukocytes and endothelial cells. Therefore, we tested whether leukocytes or endothelial cells were the targets for An-2 and Gu-4. We found by an *in vitro* binding assay that FITC-labeled An-2 and Gu-4 specifically bound to the leukocytes of rats, but neither to RLMVECs nor to NIH3T3 cells (Fig. 7). These results suggested that An-2 and Gu-4 targeted leukocytes.

Conjugation kinetics of An-2 and Gu-4 with leukocytes

To further identify the subsets of leukocytes bound to An-2 and Gu-4, we performed FACS analysis of rat blood in the presence of An-2 and Gu-4. The results of the conjugation showed that both An-2 and Gu-4 markedly bound to three different rat leukocyte subsets, *i.e.*, lymphocytes, neutrophils and monocytes, but the binding capacity of the latter two, especially neutrophils, was much higher than that of lymphocytes. The results also showed that the amount of Gu-4 conjugating with leukocytes was more than that of An-2, correlating with the more potent effects of Gu-4. Interestingly, the binding capacity of neutrophils and monocytes with these sugars was significantly enhanced in rats with burn shock ($P<0.05$) (Fig. 8), while there was a slight, but statistically insignificant increase of the binding capacity of lymphocytes isolated from burned rats.

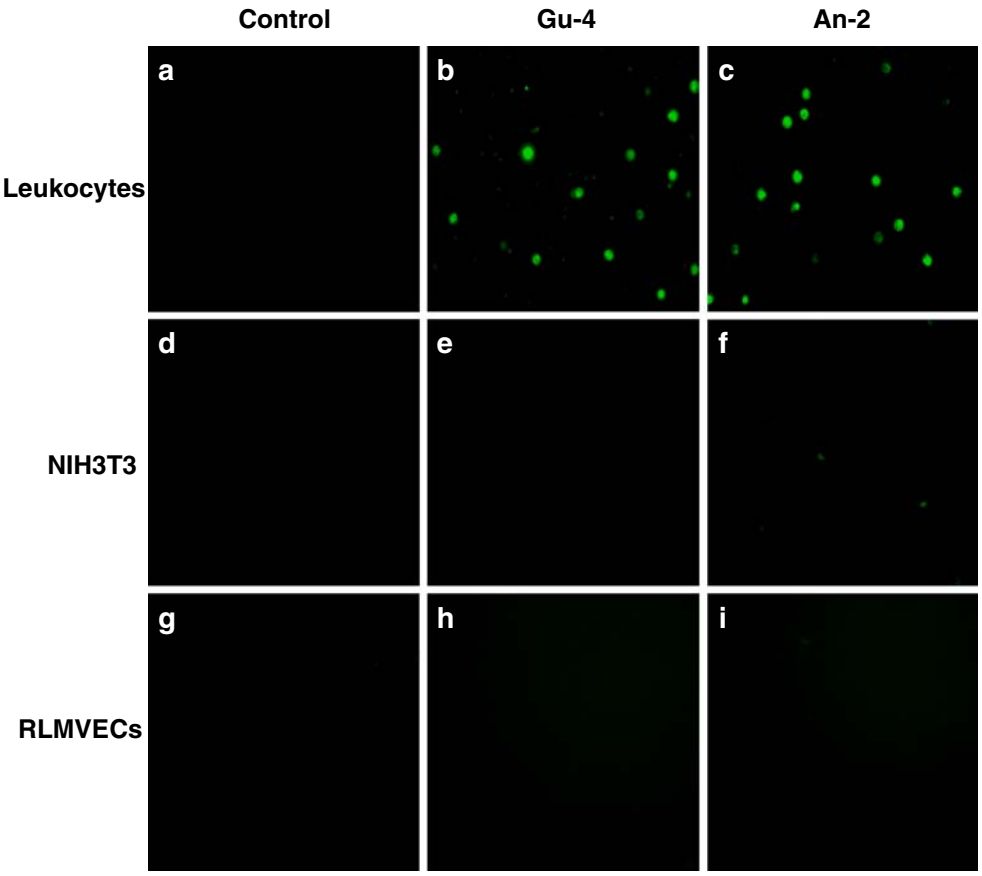

Fig 7 Selective conjugation of An-2 and Gu-4 to leukocytes of rats. Leukocytes from rats (**a**–**c**), NIH3T3 cells (**d**–**f**) and RLMVECs (**g**–**i**) were incubated with the FITC-labeled control oligosaccharide YAn-1 (**a**, **d**, **g**), YAn-2 (**c**, **f**, **i**) or YGu-4 (**b**, **e**, **h**) for 10 min and observed under fluorescence microscope. An-2 (**b**) and Gu-4 (**c**) showed a selective conjugating activity with leukocytes of rats, but neither NIH3T3 cells nor RLMVECs. Three independent experiments were performed with a comparable result

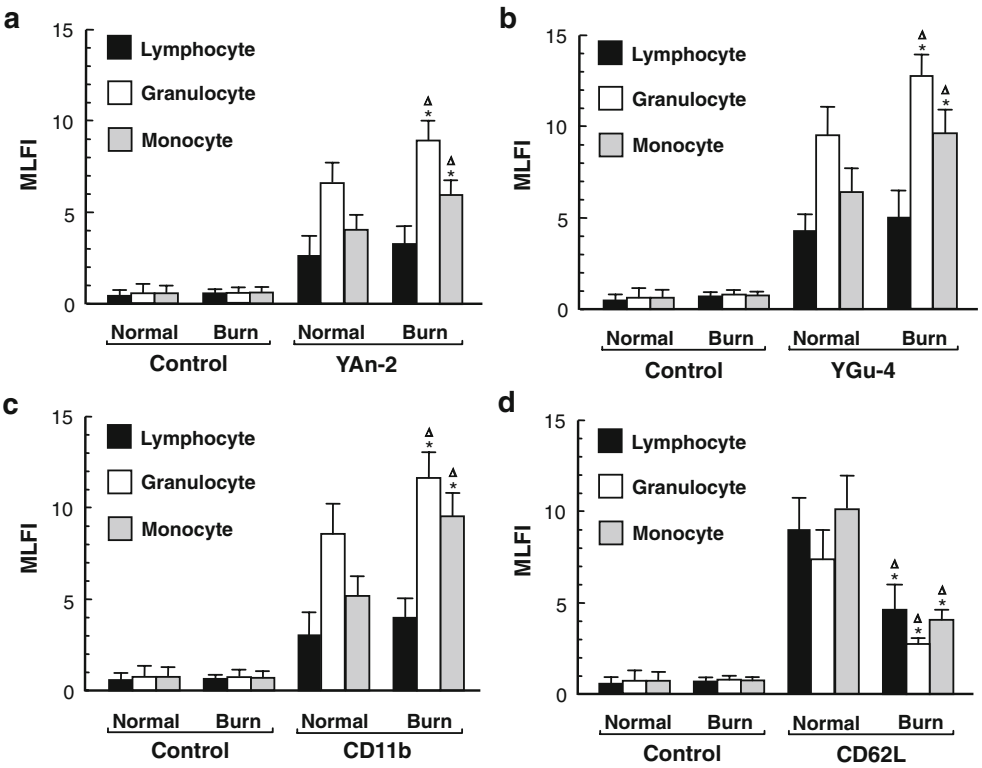

Fig 8 Conjugation profiles of An-2 and Gu-4 are similar to that of CD11b expressed on the leukocytes of rats. Leukocytes isolated from normal or burned rats were incubated with a FITC-labeled oligosaccharide or a PE-labeled monoclonal antibody. Mean logarithmic fluorescence intensity (MLFI) of leukocytes incubated with YAn-2 (**a**), YGu-4 (**b**), PE-labeled CD11b (**c**) or CD62L (**d**) monoclonal antibodies were quantitated by FACS. An unrelated FITC-labeled oligosaccharide or a PE-labeled monoclonal antibody was used as controls. The average of three independent experiments was displayed. *$P<0.05$ *vs*. normal rat group; $\Delta P<0.05$ *vs*. lymphocyte group

The expression pattern of CD11b on leukocytes correlated with the conjugation profiles of An-2 and Gu-4

To elucidate the molecular mechanism of the anti-adhesion activity of An-2 and Gu-4, the expression of two major adhesion molecules on leukocytes, CD11b and CD62L, was examined by flow cytometry. The results showed that, in normal rats, CD11b was expressed mainly on neutrophils and monocytes, and in lower amounts on lymphocytes. In rats subjected to burn shock, the expression of CD11b was significantly enhanced on neutrophils and monocytes, but not on lymphocytes (Fig. 8c). In contrast, CD62L was expressed at a high level on all subsets of leukocytes from normal control rats, but under shock conditions, CD62L expression was markedly down-regulated on all leukocytes (Fig. 8d). Notably, the expression pattern of CD11b on leukocytes correlated with the conjugation profiles of An-2 and Gu-4.

CD11b as the target molecule for An-2 and Gu-4

The similarity between the expression pattern of CD11b and the conjugation profiles of the lactosyl derivatives suggested that CD11b might be the target molecule for the conjugation of An-2 and Gu-4. Therefore, we performed a competitive binding assay by FACS and found that with increasing concentrations of CD11b mAb, the mean logarithmic fluorescence intensity of An-2 or Gu-4 on all three subsets of leukocytes decreased gradually (Fig. 9a,b). At the highest concentration of CD11b mAb (50 μg/ml) in this experiment, the conjugation of An-2 and especially Gu-4 was almost totally blocked. However, the conjugation of An-2 or Gu-4 was not significantly affected by the addition of an unrelated antibody in the control group (Fig. 9c,d).

In order to give an overview of the results above, the neutrophils conjugated with FITC-labeled An-2 or Gu-4 were analyzed as scatter plots (Fig. 10). The positive cell population binding An-2 or Gu-4 gradually shifted to the left and upward following the increase of the concentration of CD11b mAb, while the control antibody failed to show any significant effect on the cellular binding activity of the oligosaccharides. The monocytes behaved similarly to neutrophils in the competitive binding assay (data not shown).

Colocalization of CD11b and Gu-4 or An-2 on leukocyte

To provide further evidences of CD11b as a binding target for the oligosaccharides, double fluorescence staining was performed to show the colocalization of Gu-4 or An-2 with CD11b on the surface of rat leukocytes. The results of laser confocal microscopy showed that the positive fluorescence staining of Gu-4 mainly appeared on the membrane region of leukocytes, with a similar distribution pattern of CD11b on the cell membranes (Fig. 11b–d). Moreover, burn shock significantly enhanced the expression of CD11b on rat leukocytes, while the fluorescence intensity of Gu-4 bound on the cell membrane was also greatly increased (Fig. 11f–h).

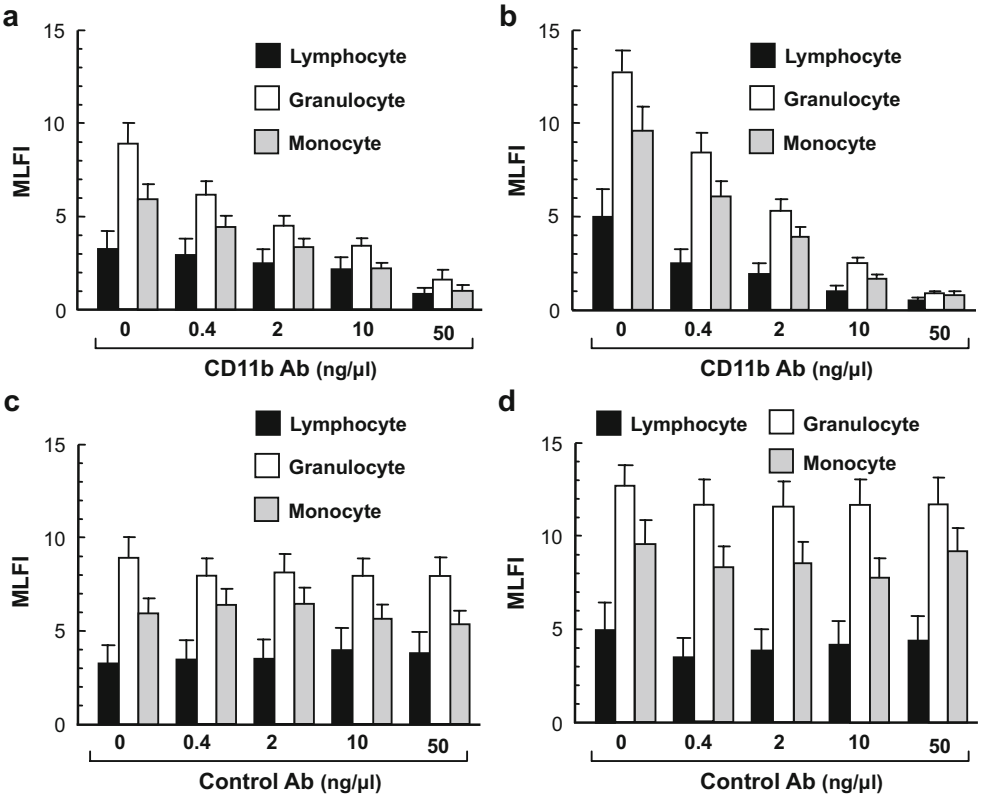

Fig 9 Dose-dependent inhibition on the conjugation of An-2 and Gu-4 with leukocytes from rats by a monoclonal antibody of CD11b. Leukocytes isolated from rats with burn shock were incubated with a FITC-labeled oligosaccharide, YAn-2 (**a**, **c**) or YGu-4 (**b**, **d**) and a PE-labeled CD11b (**a**, **b**) or a control antibody (**c**, **d**) with a concentration of 0, 0.4, 2, 10 or 50 ng/μl for a competitive binding assay. Different populations of leukocytes were identified by staining and flow cytometry as described in "Materials and methods". The results represent three independent experiments

Interestingly, the intensity of fluorescence on lymphocytes, which are smaller, was much weaker than that on the larger neutrophils and monocytes. There was no positive fluorescence staining to be found on the control, NIH3T3 cells, which were subjected to the same staining procedure (Fig. 11j–l). Similarly, An-2 also showed a colocalization with CD11b on the membrane of leukocytes (data not shown).

Gu-4 and An-2 targeting CD11b is dependent on its C-terminal lectin domain

To further characterize the domain of CD11b binding to Gu-4 and An-2, we expressed the full length or lectin domain-deficient CD11b in NIH3T3 cells by transient transfection. We found by immunofluorescence microscopy that Gu-4 showed selectively positive staining on NIH3T3 cells expressing full-length CD11b, in the form of either monomer (Fig. 12a–d) or heterodimer with CD18 (Fig. 12e–h), but neither the cells deficient on CD11b expression (Fig. 12q–t) nor those expressing the lectin domain-deficient form of CD11b (Fig. 12i–p). Convincingly, the FITC-labeled control oligosaccharide YAn-1 failed to show positive staining of green fluorescence on the cell membrane under the same condition. Similarly, An-2 also showed a positive staining on the CD11b-expressing cells, but not the cells expressing lectin domain-deficient CD11b (data not shown). All the results indicate that the C-terminal lectin domain of CD11b is a key structure for Gu-4 or An-2 targeting CD11b on the cell membrane.

Discussion

Severe or irreversible burn shock has long been a tough clinical problem and so far there is no effective therapeutic regimen, partially because the underlying mechanisms remain unclear. It is well established that inadequate blood perfusion plays a key role in organ damage and the mortality of shock [5]. In addition to the traditional approaches to improve the blood perfusion of tissues, including volume replacement and vasoactive drug application, interference with the highly increased interactions between leukocytes and endothelial cells has proven effective to resolve insufficient perfusion of the microcirculation [5, 22].

In this study, the *in vitro* flow chamber analysis showed that administration of An-2 and Gu-4 markedly blocked the adhesion of leukocytes to endothelial cells, which gives a reasonable explanation for the *in vivo* therapeutic effects of these two synthetic sugars on the rats with burn shock.

There are three major categories of adhesion molecules expressed on leukocytes and endothelial cells, *i.e.*, selectins, integrins and immunoglobulin superfamily members [4, 5, 34]. Since An-2 and Gu-4 specifically coupled to leukocytes, we predicted that the adhesion molecules expressed on leukocytes, such as L-selectin (CD62L) and

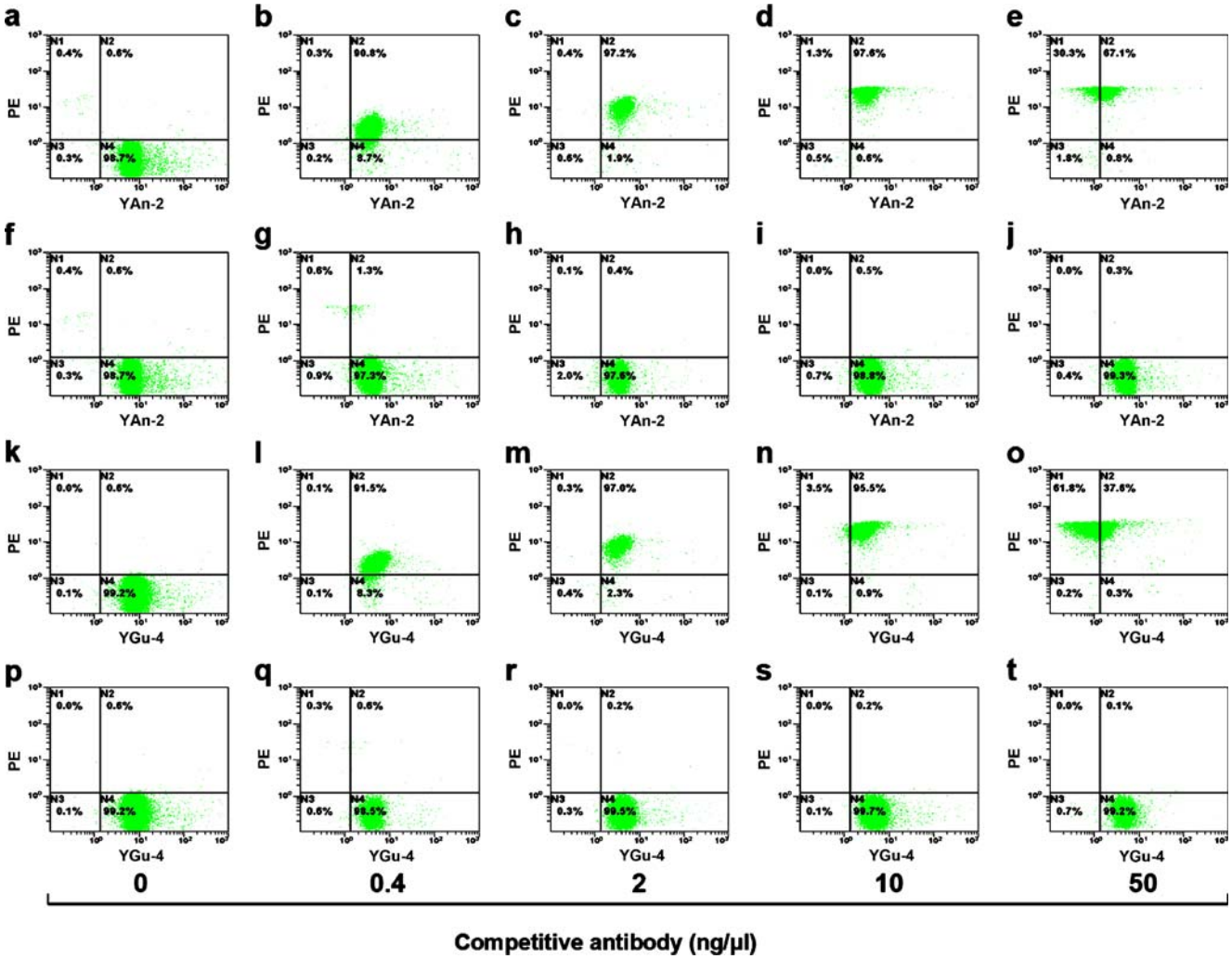

Fig 10 Dot plots of granulocytes in the competitive binding assay of YAn-2 or YGu-4 by CD11b mAb. Granulocytes were identified and gated by forward- and side-light scattering characteristics represented by MLFI using FACS assay. Leukocytes isolated from rats with burn shock were incubated with a FITC-labeled oligosaccharide, YAn-2 (**a**–**e**) or YGu-4 (**k**–**o**) and a PE-labeled CD11b (**a**–**e**, **k**–**o**) or a control antibody (**f**–**j**, **p**–**t**) with a concentration of 0, 0.4, 2, 10 or 50 ng/μl for a competitive binding assay, respectively. Three independent experiments were performed with comparable results, and one result of these experiments was displayed

β2 integrin, might be their molecular targets, while adhesion molecules mainly expressed on endothelial cells, such as P-selectin (CD62P) and E-selectin (CD62E), could be ruled out. Using flow cytometry, we found that the binding activities of the oligosaccharides with neutrophils or monocytes were much stronger than that with lymphocytes, and interestingly, the oligosaccharide conjugation kinetics with leukocytes was consistent with the increased expression pattern of CD11b, but not CD62L. Further competitive binding assay and confocal microscopy observation data led us to conclude that CD11b was a molecular target for both An-2 and Gu-4.

It is well established that CD11b is highly expressed in terminally differentiated myeloid cells, including granulocytes, monocytes, and macrophages [35, 36]. In addition, CD 11b is also expressed on a small subset of $CD5^+$ B cells [37] and $CD8^+$ T cells [38, 39], as well as a very small subset of $CD4^+$ T cells [40]. The expression of CD11b is highly regulated in myeloid cells. Unlike CDlla/CD18, CDllb/CD18 on the cell membrane of granulocytes and monocytes can be rapidly upregulated by translocation from the intracellular pool to the cell surface in response to cell activation [41, 42]. Proinflammatory factors, such as tumor necrosis factor-α (TNF-α), C5a and leukotriene B4, chemoattractants (IL-8 and formyl-methionyl-leucyl-phenylalanine) and some growth factors (platelet-derived growth factor) were reported to be potent stimuli that upregulate the expression of CD11b [41–43]. Both clinical and experimental studies had demonstrated that numerous proinflammatory factors, such as TNF-α, IL-8 and C5a, could be induced by thermal injuries [25, 44–46]. So, the increased expression of CD11b on neutrophils and mono-

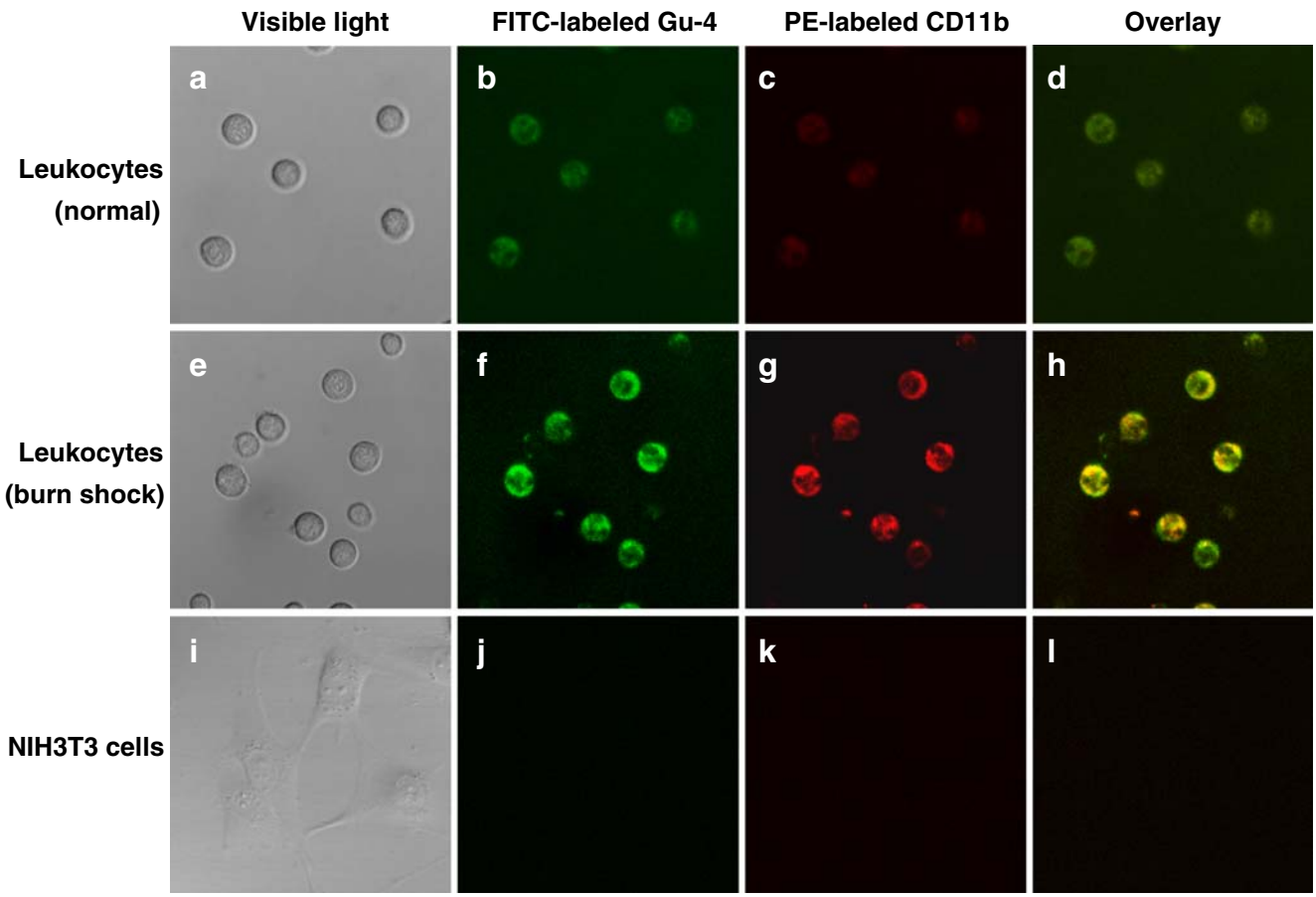

Fig 11 Colocalization of Gu-4 coupled to the leukocytes and CD11b expressed on the membrane of leukocytes. Leukocytes isolated from normal (**a**–**d**) or burn shock rats (**e**–**h**) and NIH3T3 cells (**i**–**l**) were subjected to double fluorescence staining with FITC-labeled Gu-4 and PE-labeled CD11b mAb and observed by laser confocal microscopy. **a**, **e**, **i**, Visible light; **b**, **f**, **j**, FITC-labeled Gu-4; **c**, **g**, **k**, PE-labeled CD11b mAb; **d**, **h**, **l**, merged images of Gu-4 and CD11b double fluorescence staining. Original magnification, ×1,000

cytes might be explained by stimulation of the proinflammatory factors induced by severe burn injury.

CD11b is a subunit of αMβ2 integrin, also known as Mac-1 or CD11b/CD18, on the surface of leukocytes, which has been characterized as both an adhesion molecule and a receptor for type 3 complement (CR3) [43, 47]. There are two functional domains within CD11b, the "inserted" domain (I-domain) at the *N*-terminus and the C-terminal lectin domain that plays a pivotal role for ligand binding. Upon cell activation, the I-domain is induced to form a high affinity metal ion-dependent site for the binding of protein ligands such as ICAM-1, C3 and fibrinogen. It was also reported that ligation of β-glucan with a lectin domain within the C-terminus of CD11b induces a high affinity conformational change of the I-domain [48, 49]. Other lines of evidence indicate that leukocyte adhesion *via* CD11b/CD18 binding to ICAM-1 on endothelial cells requires the formation of membrane complexes between CD11b/CD18 and GPI-anchored urokinase plasminogen activator receptor (uPAR). Sugars such as *N*-acetyl-D-glucosamine (NADG) inhibit adhesion between leukocytes and endothelial cells by disrupting complex formation [50], suggesting that a lectin-like interaction may be involved in the uPAR-dependent adhesion.

Although the structures of An-2 and Gu-4 are different from that of the saccharides reported to interact with CD11b, our data demonstrated that the receptor-ligand mediated cell adhesion was blocked by specific conjugation of An-2 or Gu-4 with CD11b, indicating that inhibition of the interaction between CD11b and its ligands by An-2 and Gu-4 may be achieved through a novel intramolecular regulation mechanism. Functional studies have shown that receptor–ligand interaction is regulated by an intracellular energy transfer mediating a conformation change [48, 50]. Based on our and others' findings, we propose that coupling of An-2 or Gu-4 to CD11b was mediated by an interaction with the lectin domain at the C-terminus of CD11b; the binding of An-2 and Gu-4 with the lectin domain either directly maintain the I-domain of CD11b at a low affinity state or disrupt the interaction between CD11b and uPAR like

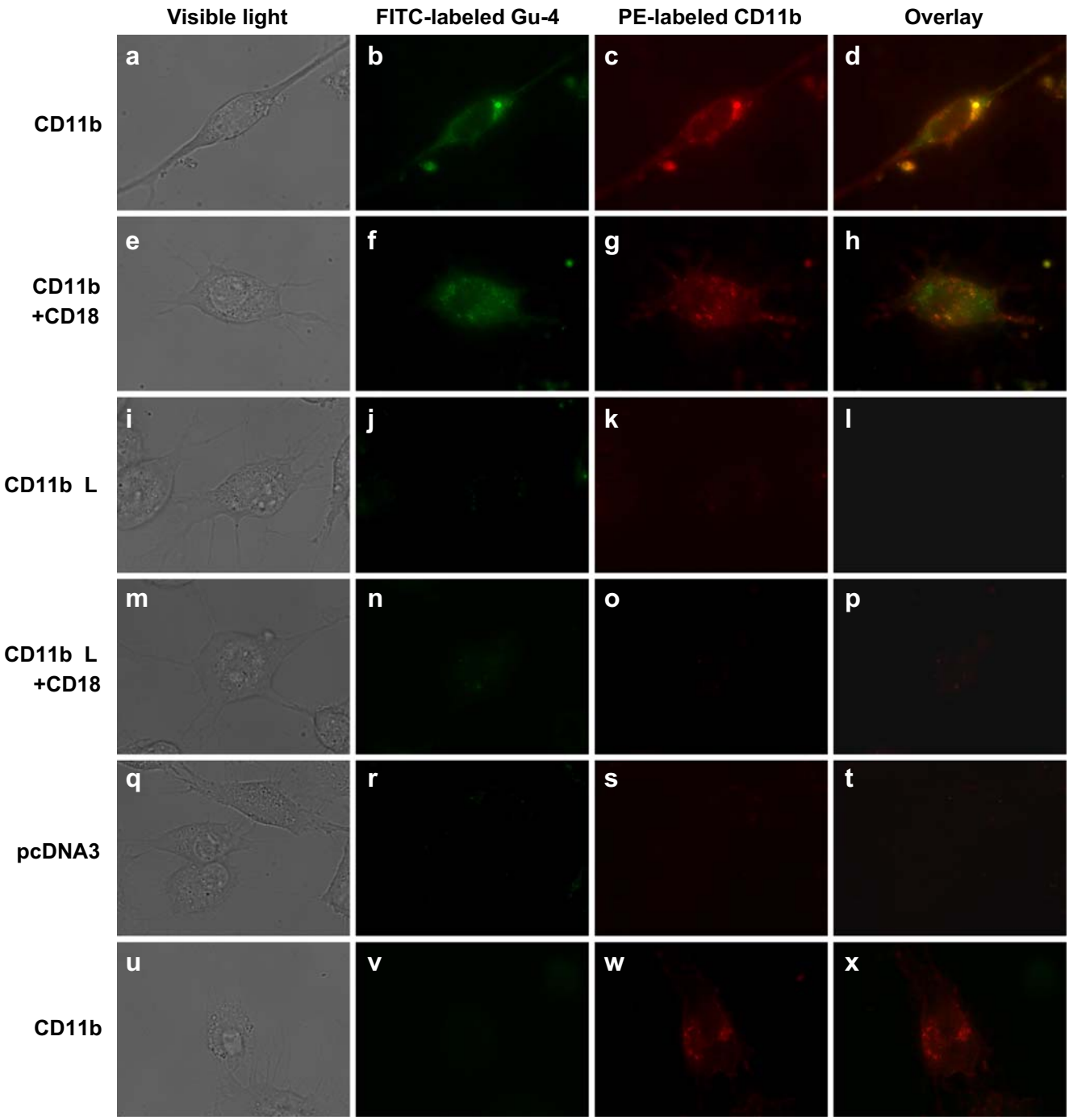

Fig 12 Lectin domain dependent interaction between Gu-4 and CD11b on the membrane of NIH3T3 cells. After being transfected with pcDNA3/CD11b (**a**–**d**; **u**–**x**), pcDNA3/CD11b plus pcDNA3/CD18 (**e**–**h**), pcDNA3/CD11bΔL (**i**–**l**), pcDNA3/CD11bΔL plus pcDNA3/CD18 (**m**–**p**) or equal amount of pcDNA3 vector, NIH3T3 cells were subjected to double fluorescence staining with the FITC-labeled saccharide YGu-4 (**a**–**t**) or YAn-1 (**u**–**x**) as control and PE-conjugated CD11b mAb, and observed under fluorescence microscope. Original magnification, ×1,000

NADG. However, whether conjugation of An-2 and Gu-4 with CD11b interferes with high affinity conformation change of I-domain or with formation of CD11b/CD18/uPAR complexes needs to be clarified in future studies.

The dose-dependent inhibition of An-2 or Gu-4 binding with CD11b by CD11b mAb might be explained in two ways: one is that the oligosaccharides and the CD11b monoclonal antibody recognize the same epitope that plays

a key role in the interaction of CD11b with its ligands; the other is that the CD11b monoclonal antibody recognizes an epitope other than that interacting with An-2 or Gu-4, but the conjugation with the antibody brings about a conformational change unfavorable for the binding of the bioactive oligosaccharides. Our results with DNA recombination technology further demonstrated that Gu-4 and An-2 binding to CD11b was dependent on the C-terminal lectin domain within CD11b, and the lectin domain-deficient form of CD11b could not be well recognized by the CD11b monoclonal antibody, indicating that this antibody exerts a competitive binding activity with An-2 and Gu-4 through direct interaction with the C-terminal lectin domain.

Gu-4 was more effective than An-2 in survival, maintenance of blood pressure, protection of tissue damage of rats with burn shock and also disruption of leukocyte-endothelial cell interaction, while the control saccharide TRT-1 failed to achieve any therapeutic effect, although TRT-1 has the lactosyl residue as in An-2 and Gu-4. This phenomenon might be explained by "cluster effect", because there are four sugar groups attached to the linker of Gu-4, and two for An-2, but only one for TRT-1. It is very possible that only multi lactosyl residues linked together could bring about cluster effect by binding to CD11b, thus effectively affect the conformation of CD11b.

In conclusion, we demonstrated herein that the lactosyl derivatives, An-2 and Gu-4, have a significant therapeutic effect on rats with severe burn shock by transiently inhibiting leukocyte-endothelial cell adhesion. Furthermore, we identified CD11b on leukocytes as a specific molecular target for the oligosaccharides. It had been reported that CD11b plays an essential role in diseases associated with leukocyte adhesion [43, 48, 49] and *in vivo* inactivation of CD11b shows great protective effects on acute lung injury induced by *Escherichia coli* septicemia [51]. Our findings are important not only for identification of a novel small sugar type inhibitor for CD11b, but also for extending the window of anti-adhesion therapy for the severe burn shock.

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