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A Direct Immunoassay Assessment of Streptavidin- and N-Hydroxysuccinimide-Modified Biochips in Validation of Serological TNFα Responses in Hemophagocytic Lymphohistiocytosis

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The authors report 2 biochip platforms on gold manufactured by either nanoscale biotinylated self-assembled architectures to streptavidin surface or proteins containing free NH_2 groups to N-hydroxysuccinimide (NHS)–activated surfaces and investigated the potential application of tumor necrosis factor–α (TNFα) serodiagnosis of hemophagocytic lymphohistiocytosis (HLH). Interactions of TNFα antigen and TNFα antibody on the biochips were optimized using an indirect immunofluorescence method. Variation coefficients were 1.87% to 4.56% on the streptavidin biochip and 5.03% to 8.64% on the NHS biochip. The correlation coefficients (*r*) in TNFα and TNFα antibody assays in HLH patients between the 2 biochip formats were 0.9623 and 0.9386 and the concordance frequencies were 92.2% and 96.1%, respectively. To detect plasma TNFα-receptor complexes (TNFR1 and R2) in HLH, a biochip assay strategy was developed. Plasma levels of TNFα, TNFα antibody, and TNFα-receptor complexes (TNFR1 and R2) were detected in plasmas from 42 HLH cases using streptavidin biochips. Frequencies of the biomarkers in the plasmas were 40.5% (17/42) for TNFα, 30.9% (13/42) for TNFα antibody, 28.6% (12/42) for TNFα–receptor 1 complex, and 26.1% (11/42) for TNFα–receptor 2 complex, respectively. The streptavidin biochip format was more sensitive than the NHS surface and was demonstrated to be a valuable tool to identify individual biomarker molecules and molecular complexes in sera and cell lysates and to track therapeutic progress of patients. (*Journal of Biomolecular Screening* XXXX:xx-xx)

Key words: biochip, streptavidin, N-hydroxysuccinimide, TNFα, hemophagocytic lymphohistiocytosis, protein assay

INTRODUCTION

DEVELOPMENT OF BIOCHIP TECHNOLOGIES OFFERS THE POSSIBILITY to analyze protein profiling, peptide mapping, protein interactions, and identification of proteins in a single assay.[1-7] Protein biochips provide a large pool of data at a low cost and with a small amount of serum and body fluids necessary for testing, which can be applied not only to protein function studies, production screening of antibodies, and recombinant proteins[6,8,9] but also to discovery of proteins implicated in disease and potential drug targets, as well as rapid detection or diagnosis of disease.[9] Most of the current analyses of protein-related interactions on biochips are based on the formats that were earlier established in the case of DNA arrays.[10,11] Coupling of the molecules to the biochip surface should be high yield and have high sensitivity and stability.[10,12] However, some intrinsic shortcomings in fabrication technologies of protein biochips affect their development and application because it is difficult to maintain active forms of capture proteins on a solid surface in such a way that their 3-D structures, functionalities, and binding sites are retained and to control the biocompatibility of the surface and the orientation of the attached biomolecules.[10] To avoid the bottlenecks, we tested various chemical surfaces on arrays with a variety of coupling chemistries, such as amine,[13] aldehyde,[14] poly-L-lysine,[4] epoxy,[15] agarose,[16] polyacrylamide,[17] and nitrocellulose.[18] An optimal surface modification enables localizing fine architecture, increasing binding capacity of proteins, improving signal uniformity, and extending detection sensitivity on biochips.

Self-assembled monolayers are molecular architectures that are formed spontaneously upon the interaction of a surface-active head group with an appropriate substrate in solution.[12] Thiols and disulfides are the most commonly used reactive molecules on noble metal substrates such as gold and silver. The surface-active head groups are usually coupled to an alkyl or a derivatized alkyl chain compound. The terminal end of the alkyl chain molecules can also be derivatized to yield a number

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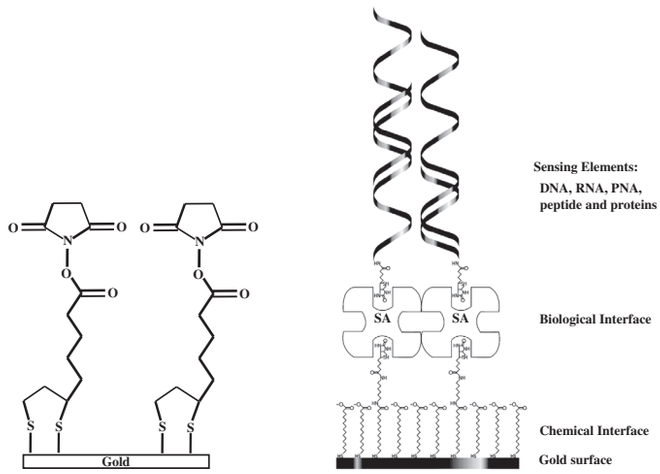
of active groups such as -OH, -NH_2, -COOH, -COOR, and so on, thereby producing surfaces with different interfacial properties.[10] The thiol molecules adsorb readily from solution onto the gold. If the alkyl chain is long enough, these architectures are very stable and show a molecular orientation nearly perpendicular to the surface. By using thiol molecules with different tail groups, the resulting chemical surface functionality can be varied within wide limits.[10,12] Protein probes to be immobilized supply the following functionalities in the side chains of the polypeptide backbone: -SH (cysteine), -NH_2 (lysine, arginine), -COOH (asparagine, glutamine), -OH (serine), Ph-OH (Ph = phenyl, tyrosine), and imidazole (histidine), which can be used for a direct chemical coupling reaction to specially prepared biochip surfaces. Therefore, the self-assembled monolayer technique provides such a versatile tool to prepare surfaces with good biocompatibility and customized properties with respect to solutions for many detection problems.[19]

One of the most widespread biochip manufacturing techniques is activation of the biochip surface with ester compounds, such as N-hydroxysuccinimide (NHS). The reaction on the NHS-modified biochip surface includes formation of the intermediate active ester and the product of condensation of the carboxylic group and N-hydroxysuccinimide, which further reacts with the amine function to finally yield the amide bond.[20] These active esters show a high reactivity and selectivity toward the primary amino group (**Fig. 1A**). The NHS-esters are especially suitable for coupling of all kinds of amino-functionalized biomolecules in aqueous-buffered media because of their stability toward hydrolysis reactions. Another method that controls the orientation of the biomolecule on the biochip surface in an ordered way is provided by proteins bearing streptavidin tags, supplying the binding site of the molecule on the surface. The biotin unit of biotinylated molecules forms very stable supramolecular complexes with streptavidin on the surface by means of biotin-terminated monolayers (**Fig. 1B**) or mixed monolayers.[10] In our previous research, a gold surface suitable for the identification of protein and peptide targets has been developed.[1,6,21,22] Possibilities of practical use of NHS- and streptavidin-modified biochips in the diagnostic area and several advantages over a standard enzyme-linked immunosorbent assay (ELISA) method were evaluated separately.[6,22] However, the validations of diagnostic methods using these surfaces were done using different assay methods. An objective assessment of heterogeneity for immobilized proteins in the same assay system using 2 surface chemistries remains to be explored further.

Hemophagocytic lymphohistiocytosis (HLH) is a rare autosomal recessive disorder that is often fatal despite treatment in infancy and early childhood. HLH is caused by a dysregulation in natural killer T cell function, resulting in activation and proliferation of lymphocytes or histiocytes with uncontrolled hemophagocytosis and cytokine overproduction. Infections associated with HLH are most frequently caused by viruses, particularly Epstein-Barr virus



A NHS surface **B. streptavidin surface**

FIG. 1. (**A**) Streptavidin- and N-hydroxysuccinimide (NHS)–modified biochip surfaces. Reaction of NHS surface includes formation of the intermediate active ester and the product of condensation of the carboxylic group and N-hydroxysuccinimide, which further reacts with the amine function to finally yield the amide bond. (**B**) Coupling of polyethylene oxide–aminobiotin to 16-mercaptohexadecanoic acid is performed on gold. The biotinylated molecules form stable supramolecular complexes with streptavidin on the surface by means of biotin-terminated monolayers.

(EBV). The autoimmune mechanism might be involved in HLH pathogenesis. HLH is clinically characterized by fever, hepatosplenomegaly, cytopenias, liver dysfunction, hyperferritinemia, and hypercytokinemia, such as tumor necrosis factor–α (TNFα), interleukin-2 (IL-2), IL-6, IL-8, interferon-gamma (IFN-g), IL-10.[23] High concentrations of inflammatory cytokines may be responsible for hyperactivation of dendritic cells, leading to pancytopenia and a high risk for infections and death.[6,24-26] The diagnosis of HLH is made by seroconversion to demonstrate specific antibodies. Rapid and efficient determinations of elevated cytokines with a very small amount of serum or plasma will improve diagnosis dramatically for obvious reasons. An ELISA for cytokine detection was described.[27] However, ELISA-based tests were time-consuming and required larger quantities of both samples and reagents. Currently, advances in protein microarray technology have benefited the medical community greatly, making miniaturization and high throughput possible, getting the comparisons among 2 or more experiments more feasible, and partially overcoming the ELISA limitations in serum immunological diagnosis.[8,28,29] Primary methodological evaluations of protein biochips and commercial ELISAs in screening serum antigens and antibodies of pathogens and cytokines were performed.[6,22,28] The protein biochip technique has been used to measure mainly TNFα[30,31] and less with detecting TNFα antibody[32] in a variety of human diseases. However, investigating serological TNFα and TNFα antibody as

well as interactions of TNFα with its relevant receptors (TNFR1, CD120a and TNFR2, CD120b) in HLH by the protein biochip technique has not yet been reported so far.

Here we identify the quantitative assessment of heterogeneity for immobilized proteins on the gold surface that was modified with either streptavidin or N-hydroxysuccinimide, respectively. Thus, coupling of biotinylated or nonbiotinylated proteins to the streptavidin and NHS surfaces allows the subsequent testing of antibodies and antigens present within the plasma samples of patients with HLH. The antigen-antibody biomolecular interactions on the protein biochips were monitored by using fluorescence detection schemes. A wide range of experimental parameters such as sensitivity and reproducibility that influence the efficacy of the biochip assays was investigated, and subsequently, the potential of the protein biochips in serologically diagnosing TNFα, TNFα antibody, and TNFα-receptor complexes (TNFR1 and TNFR2) in HLH was evaluated.

MATERIALS AND METHODS

Reagents and biochips

Biotinylated and nonbiotinylated recombinant human TNFα and polyclonal goat IgG antirecombinant human TNFα antibody, as well as monoclonal antihuman TNFα receptor 1 (CD120a, p55, clone 16803) and receptor 2 (CD120b, p75, clone 22210) antibodies, were purchased from R&D Systems GmbH (Wiesbaden-Nordenstadt, Germany). Monoclonal antirecombinant human TNFα antibody (clone 2H4) was purchased from Roche Diagnostics GmbH (Mannheim, Germany). Rabbit fluorescein-conjugated IgG fractions of antimouse and antihuman IgG F (ab′)2 were supplied by Rockland Company (Biomol GmbH, Hamburg, Germany). Streptavidin- and NHS-modified biochips for protein assays were supplied by Thermo Electron Corp (Division Ulm, Germany). Briefly, glass slides, covered with a 0.1-μm 24-carat gold layer, were coated with a 50-μm thin hydrophobic Teflon film that creates hydrophilic microwells (1.5 mm diameter) to prevent nonspecific interaction with the biochip background. A self-assembled monolayer was formed after incubation in 16-mercaptohexadecanoic acid solution. Subsequently, the monolayer was biotinylated with biotin–polyethylene oxide (PEO)–amine (Pierce, Rockford, IL) and then saturated with recombinant streptavidin (Roche). For preparation of N-hydroxysuccinimide-modified biochips, the same format of biochips was treated with DL-α-lipoic acid (Sigma, St. Louis, MO) and then N-hydroxysuccinimide (Sigma) in the presence of N, N′-dicyclohexyl-carbodiimide (DDC, Sigma) to assist the carbodiimide coupling.

Blood sample collection

Plasma samples from 71 patients with HLH were collected conventionally. HLH diagnosis was based on diagnostic guidelines developed by the Histiocyte Society.[24] Eighteen plasma samples were individually selected from 1 HLH patient within 16 months. The patient was treated during the period of the disease under the application of chemotherapy and following anti-CD25 therapy (Zenapax, CD25 specific antibody, 1.0 mg/kg, Roche). Four plasma samples from the patient's healthy sibling at different sampling times were used as a control.

Estimation of the amount of immobilized molecules on streptavidin- and NHS-modified surfaces

To optimize the protein-coating concentrations on biochips, we dissolved biotinylated and nonbiotinylated recombinant human TNFα antigens in the phosphate-buffered saline with Tween-20 (PBST)/bovine serum albumin (BSA) buffer: 120 mM NaCl, 2.7 mM KCl, and 10 mM phosphate buffer (pH 7.4, Invitrogen, Karlsruhe, Germany); 0.1% Tween-20 (v/v, Sigma, Steinheim, Germany); and 0.1% BSA (wt/v, Sigma, Steinheim, Germany). Equal amounts of protein molecules of the same target specificity were then immobilized onto streptavidin- and NHS-modified biochips, respectively, vertically in columns at 8 different concentrations (*y*-axis) ranging from 0.063, 0.125, 0.25, 0.5, 1.0, 2.0, 4, and 8.0 μg/mL of TNFα antigen (1 μL/spot). PSBT-BSA buffer was used as a negative control in this experiment. All surface immobilization and the subsequent steps were done in humid chambers to prevent an aqueous evaporation effect. The procedures of protein coatings on biochips were performed at room temperature for 3 h. The biochips were rinsed with sterile water briefly and then in PBST buffer for 2 min, 3 times, respectively. After drying with a stream of nitrogen, the antigen biochips were incubated with monoclonal TNFα antibody, horizontally in lines at 12 different concentrations (*x*-axis) ranging from 0, 0.049, 0.098, 0.195, 0.39, 0.78, 1.56, 3.12, 6.25, 12.5, 25.0, and 50 μg/mL in PBST-BSA buffer (pH 7.4, 1 μl/spot) at room temperature for 1 h. Following rinsing and drying, the reaction complex on biochips was detected by 50 μg/mL of fluorescein (FITC)–conjugated rabbit antimouse IgG antibody in PBST-BSA buffer (pH 7.4) at room temperature for 60 min in the dark. The biochips were washed in PBST buffer and then covered with a cover glass. Arrays were imaged using a fluorescence scanner (XNA ScanPro 20 microarray scanner, Thermo Hybaid, Middlesex, UK) at 488 nm for FITC. The spatial resolution of 16 bits per pixel and a 50-μm pixel size were chosen for scanning the biochips. Image data were quantified with the microarray analysis software, AIDA 2.11 (Raytest, Straubenhard, Germany). The fluorescence intensities obtained from the spots immobilized with PBST-BSA buffer were used as background value. The fluorescence signals are expressed as arbitrary units across a 1-mm area^2 (AU/mm^2).

For sorting of appropriate polyclonal capture antibody-coating concentrations, a similar experiment to the antigen optimization was performed—that is, biotinylated and nonbiotinylated polyclonal goat antihuman TNFα antibodies were immobilized onto streptavidin- and NHS-modified biochips, respectively, vertically in columns at 8 different concentrations (*y*-axis) ranging from

0.78, 1.56, 3.12, 6.25, 12.5, 25.0, 50.0, and 100 μg/mL in PBST-BSA buffer (pH 7.4, 1 μL/spot), and then the antibody biochips were incubated with TNFα antigen horizontally in lines at the same 12 different concentrations (*x*-axis) as described above in TNFα antigen assay. Antibody-antigen complexes were detected with 50 μg/mL of monoclonal antihuman TNFα antibody in PBST-BSA buffer (pH 7.4). The monoclonal TNFα antibody was then detected by 50 μg/mL of FITC-conjugated rabbit antimouse IgG antibody in PBST-BSA buffer (pH 7.4).

Immunocompetent optimizing of proteins on biochips

In this study, 2 and 50 μg/mL of proteins were optimized as immobilization concentrations on streptavidin- and NHS-modified biochips, respectively, unless indicated elsewhere. TNFα antigen-coated biochips were incubated with mouse antihuman TNFα-specific antibody (50 μg/mL) at room temperature for 15, 30, 45, 60, 90, and 120 min in humid chambers, respectively, to validate the optimal interaction time. After detection with a FITC-conjugated rabbit antimouse IgG antibody (50 μg/mL), the fluorescence intensities of 12 replicates from each biochip were given as mean ± SD (AU/mm^2, *y*-axis).

The protein biochip variability of streptavidin- and NHS-modified biochip formats was tested by repeating 6 individual biochips on 6 days. The experiment was based on the TNFα antibody assay on TNFα antigen-coated biochips and then following the interaction of FITC-labeled rabbit antihuman IgG antibody. The average fluorescence signals and standard deviations from 16 spots in each dilution were calculated. The fluorescence intensities of the spots coated by PBST (pH 7.4)–0.1% BSA on each biochip were used as background control.

To validate the shelf time of protein-loaded biochips, we investigated dynamic changes of fluorescence intensities of the 2 protein biochips after being stored at 4 °C in a desiccated condition for different times (0 day, 1 day, 1 week, 2 weeks, 4 weeks, and 8 weeks). The average fluorescence intensities and standard deviations from 16 spots in each storage group were calculated. The fluorescence intensities of the spots coated by PBST (pH 7.4)–0.1% BSA at different storage times were used as background control.

Sandwich immunoassay of serological TNFα antigen on biochips

Immunoassays for detecting different molecules captured were performed on individual biochips, respectively. Streptavidin- and NHS-modified biochips were used for the TNFα assay. EDTA-treated plasma samples from patients with HLH were diluted 1:50 in PBST-BSA buffer (pH 7.4) and added onto the 2 biochips precoated by polyclonal goat IgG antihuman TNFα antibody. The biochips were incubated at room temperature for 60 min. Each point represents the result of 1 patient's plasma. Then the biochips were incubated with 50 μg/mL of mouse antihuman TNFα antibody for an additional 60 min at room temperature. TNFα antigen and antibody complexes on biochips were further detected by the FITC-conjugated rabbit antihuman IgG antibodies (50 μg/mL) at room temperature for 60 min. Average fluorescence values of the plasmas from the healthy individuals and PBST-BSA buffer were calculated as negative and background controls, respectively. Fluorescence values of 2 times of mean and 2 SD over negative control were evaluated to be positive.

Serological antibody immunoassay on biochips

Streptavidin- and NHS-modified biochips were used for TNFα antibody assay. Patients' samples tested were diluted 1:50 in PBST-BSA buffer. The serological assay was performed on TNFα antigen-immobilized biochips at room temperature for 60 min. The bindings of specific antibodies with TNFα antigen on the biochips were respectively detected by the FITC-conjugated rabbit antihuman IgG antibody (50 μg/mL) at room temperature for 60 min. Average fluorescence values of the plasmas from the healthy individuals and PBST-BSA buffer were calculated as negative and background controls, respectively.

Sandwich immunoassay of serological TNFα-receptor complexes on biochips

The streptavidin-modified biochip was used for the TNFα-receptor complex assay. EDTA-treated plasma samples from 42 patients with HLH were diluted 1:50 in PBST-BSA buffer (pH 7.4) and added onto the streptavidin-modified biochips immobilized by polyclonal goat IgG antihuman TNFα antibody. The biochips were incubated at room temperature for 60 min and then with 50 μg/mL of mouse antihuman TNFα receptor 1 or 2 antibody, respectively, for additional 60 min at room temperature. TNFα-receptor complexes on biochips were further detected by the FITC-conjugated rabbit antimouse IgG antibody (50 μg/mL) at room temperature for 60 min.

Blocking assays

For blocking assays, 4 individual plasmas with positive TNFα and TNFα antibody responses were pretreated with standard TNFα antibody at the concentrations of 0.05, 0.5, and 5 μg/mL and with TNFα antigen at 0.1, 1, 10, and 100 μg/mL, respectively, at room temperature for 60 min and then followed by the biochip assays for plasma TNFα and TNFα antibody, respectively. Healthy plasma was used as negative control.

RESULTS

Quantification of TNFα antigen and antibody on streptavidin- and NHS-modified biochips

Figure 2 shows that the fluorescence intensities (quantified as AU/mm^2) in each group increased with higher concentrations of

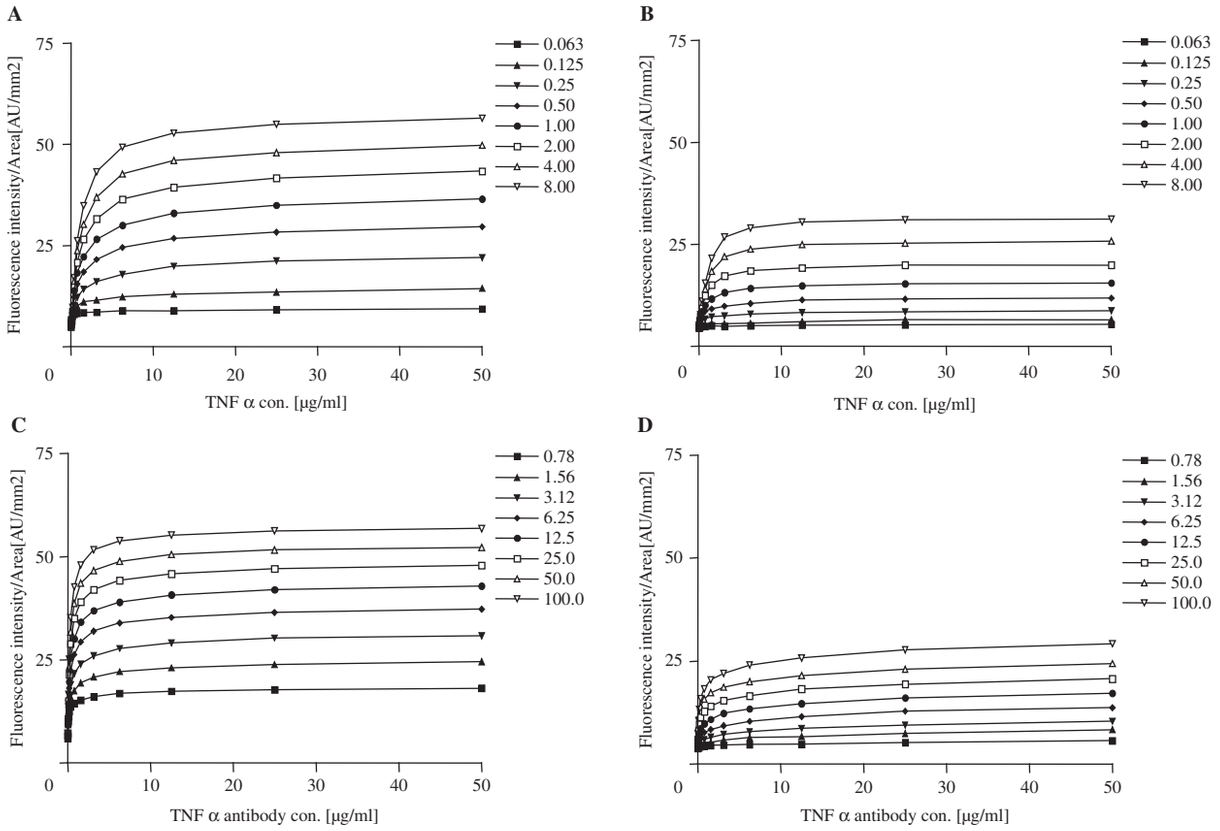


FIG. 2. Determination of dynamic ranges of human recombinant tumor necrosis factor–α (TNFα) antigen and TNFα antibody on streptavidin- and N-hydroxysuccinimide (NHS)–modified biochips. (**A**) Biotinylated and (**B**) nonbiotinylated TNFα antigen were coated in a horizontal direction in 8 different concentrations (0.063-8 μg/mL, 1 μL/spot, *y*-axis), whereas monoclonal antibody was added vertically in columns in 12 different concentrations (0-50 μg/mL, 1 μL/spot, *x*-axis). The antigen-antibody complexes were detected by fluorescein (FITC)–conjugated rabbit antimouse IgG antibody (50 μg/mL). The fluorescence signals are expressed as arbitrary units per mm^2 (AU/mm^2). For determination of coating polyclonal antihuman TNFα antibody on biochips, (**C**) biotinylated and (**D**) nonbiotinylated polyclonal goat antihuman TNFα antibodies were immobilized in a horizontal direction in 8 different concentrations (0.78-100 μg/mL, *y*-axis). The antibody layers were subsequently incubated with rhTNFα antigen in columns in 12 different concentrations (0-50 μg/mL, *x*-axis). Antibody-antigen complexes were detected by monoclonal TNFα antibody and then FITC-conjugated rabbit antimouse IgG antibody.

immobilized recombinant protein on biochips, presenting a linear correlation in both streptavidin- and NHS-modified antigen biochips. Fluorescence values of 2 times of mean and 2 SD over background value were evaluated to be positive. The detection limits of TNFα antigen were identified at the concentrations of 0.25 μg/mL on the streptavidin-modified biochip (**Fig. 2A**) and 0.5 μg/mL on the NHS-modified biochip (**Fig. 2B**), respectively, when 0.78 μg/mL of specific antibody was added. Similarly, the detection limits of TNFα antibody were 0.78 μg/mL on the streptavidin-modified biochip (**Fig. 2C**) and 6.25 μg/mL on the NHS-modified biochip (**Fig. 2D**), respectively. In both cases, the streptavidin-modified biochip presented a more sensitive on-chip protein assay about 2 times higher than the NHS-modified biochip at the same experimental condition.

Optimization of time course of antigen and antibody interaction on biochips

To optimize the time course of the antigen and antibody interaction on biochips, we incubated TNFα-coated biochips with mouse antihuman TNFα-specific antibody (50 μg/mL) for 15, 30, 45, 60, 90, and 120 min at room temperature. The fluorescence intensity results of 12 replicates from each biochip are given as mean ± SD (AU/mm^2). The results showed that a plateau of fluorescence signal intensity from the streptavidin- and NHS-modified biochips occurred after incubation for 45 min (**Fig. 3**). The mean fluorescence intensities of signal and baseline at 45 min were 43.94 ± 1.20 and 6.15 ± 0.70 on streptavidin-modified biochips and 35.07 ± 1.05 and 6.14 ± 0.37 on NHS-modified biochips,

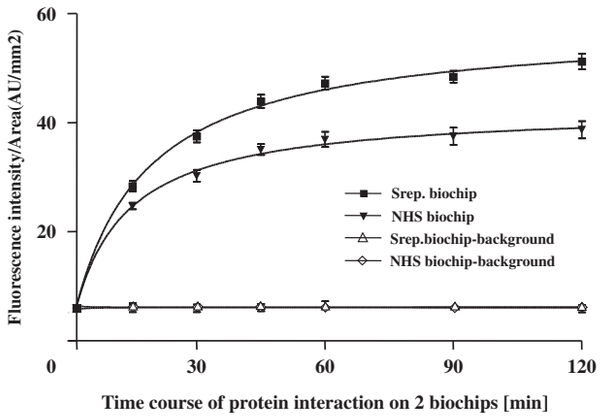


FIG. 3. Optimization of time course of antigen and antibody interaction on biochips. Tumor necrosis factor–α (TNFα)–coated biochips were incubated with mouse antihuman TNFα-specific antibody (50 μg/mL) for 15, 30, 45, 60, 90, and 120 min (*x*-axis) at room temperature. After detection with a fluorescein (FITC)–conjugated rabbit antimouse IgG antibody (50 μg/mL), the fluorescence intensities of 12 replicates from each biochip were given as mean ± SD (AU/mm^2, *y*-axis). NHS, N-hydroxysuccinimide.

respectively. Obviously, the interaction of immobilized TNFα with the specific antibody was time dependent.

Protein biochip reproducibility

The protein biochip variability was tested by repeating 6 biochips on 6 days. The average fluorescence signals and standard deviations from 16 spots in each dilution were calculated. The variation coefficient on the 6 biochips ranged from 1.87% to 4.56% on the streptavidin-modified biochip and 5.03% to 8.64% on the NHS-modified biochips (**Table 1**).

Shelf life results of on-chip proteins showed that fluorescence intensities from the protein biochips tended to decrease when stored for a longer period. However, both 12 TNFα antigen and TNFα antibody biochips could easily differentiate samples from background samples after 8 weeks of storage (**Fig. 4A,B**). At week 8, the mean fluorescence intensities and baseline levels (mean ± SD) of the biochips were 185.97 ± 14.74 versus 19.10 ± 0.92 on the streptavidin-modified biochips and 130.22 ± 12.98 versus 11.99 ± 0.92 on the NHS-modified biochips for the TNFα assay, as well as 53.31 ± 2.52 versus 13.38 ± 1.06 on the streptavidin-modified biochips and 26.82 ± 0.80 versus 11.35 ± 0.56 on the NHS-modified biochips for the TNFα antibody assay, respectively, which were 2 times of the mean values and 2 SD over the baseline levels at each time point during the shelf life.

Table 1. Protein Biochip Variability Tested by Repeating 6 Biochips on 6 Days

	Streptavidin Biochip		*NHS Biochip*	
Groups	*Means (AU/mm^2)*	*CV (%)*	*Means (AU/mm^2)*	*CV (%)*
Chip 1	57.58	3.75	22.58	7.86
Chip 2	56.69	1.87	22.45	5.08
Chip 3	57.53	3.42	23.06	5.03
Chip 4	60.19	4.56	24.28	6.41
Chip 5	59.84	3.64	26.36	6.12
Chip 6	58.53	2.49	28.76	8.64

The average fluorescence signals and standard deviations from 16 spots in each dilution were calculated. CV, coefficient of variation; NHS, N-hydroxysuccinimide.

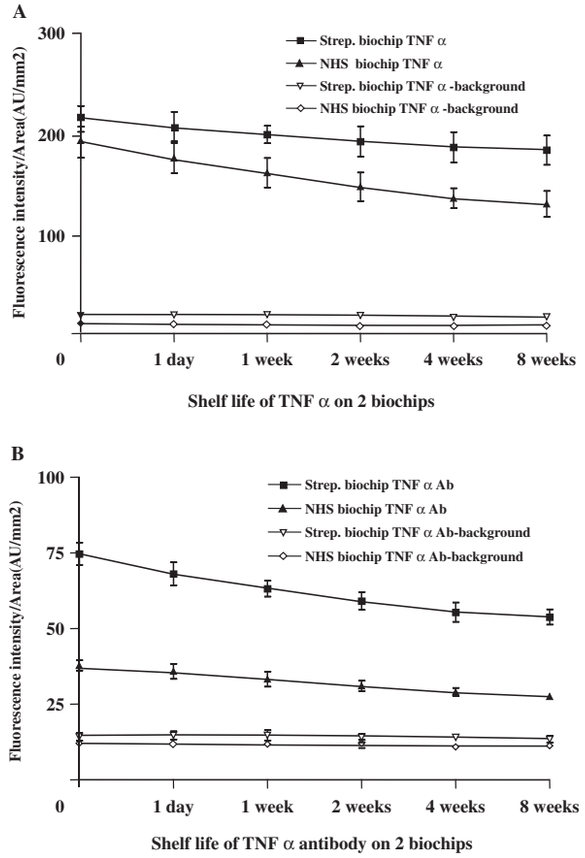


FIG. 4. Validation of shelf time of protein-loaded biochips. Dynamic changes of fluorescence intensities of (**A**) tumor necrosis factor–α (TNFα) and (**B**) TNFα antibody on streptavidin- and N-hydroxysuccinimide (NHS)–modified biochips were investigated after being stored at 4 °C in a desiccated condition for different times (0 day, 1 day, 1 week, 2 weeks, 4 weeks, and 8 weeks). The average fluorescence intensities and standard deviations from 16 spots at each storage time were given (*y*-axis).

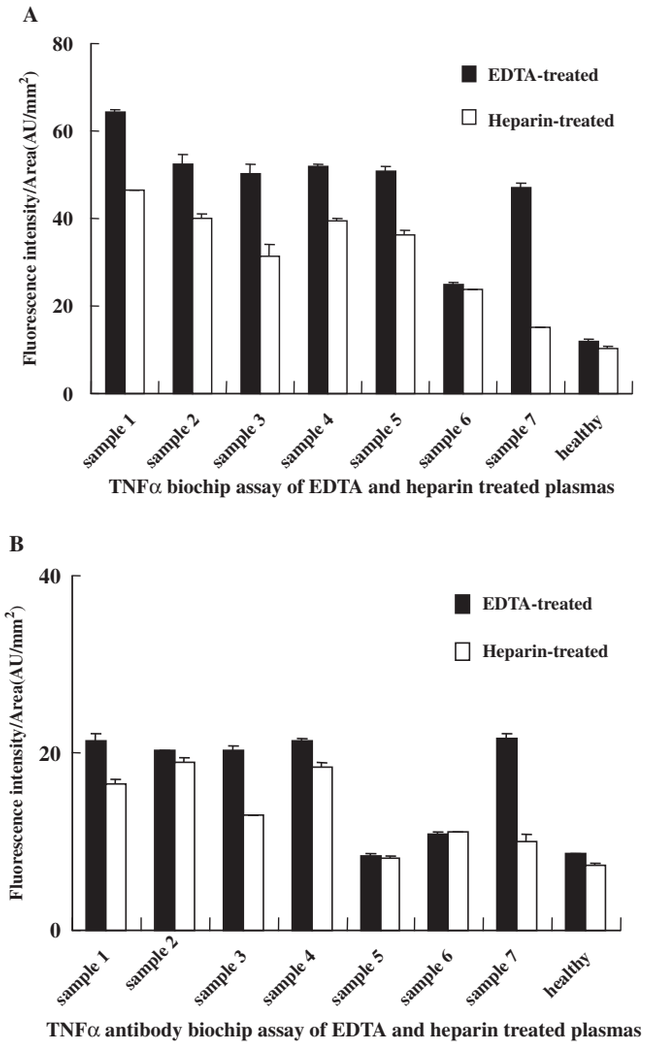


FIG. 5. Effect of EDTA and heparin pretreatment on preserving plasma (**A**) tumor necrosis factor–α (TNFα) and (**B**) TNFα antibody was investigated in 7 hemophagocytic lymphohistiocytosis (HLH) plasmas and 1 healthy plasma by the streptavidin biochip assay. The fluorescence intensities of duplicate spots from each sample are given as mean ± SD (AU/mm^2).

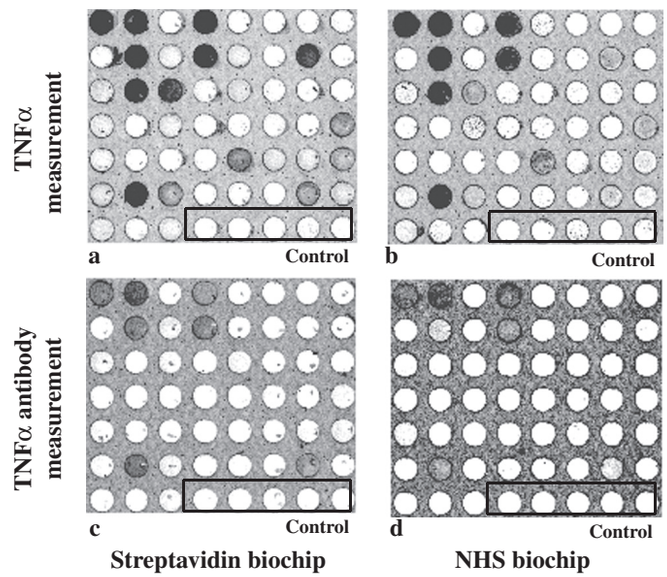


FIG. 6. Tumor necrosis factor–α (TNFα) and TNFα antibody assays of 51 samples from 37 hemophagocytic lymphohistiocytosis (HLH) patients on streptavidin- and N-hydroxysuccinimide (NHS)–modified biochips. Plasmas (1:50 dilution, 1 μL/spot) were incubated on (**A, C**) streptavidin-modified and (**B, D**) NHS-modified biochips. (**A, B**) TNFα and (**C, D**) TNFα antibody were detected, respectively. Each point represents the result of individual patient plasma. Five healthy plasmas (black frames) were used as negative controls.

Comparison of assay sensitivity of plasma TNFα and the specific antibody between streptavidin- and NHS-modified biochips

Seven pairs of either EDTA- or heparin-treated plasmas were collected from 6 HLH patients and 1 healthy individual. The effect of EDTA and heparin pretreatment on preserving plasma TNFα and TNFα antibody activities was compared on streptavidin-modified biochips. EDTA-treated plasma could preserve more native activities of TNFα and TNFα antibody than the heparin-treated one (**Fig. 5A,B**) in the biochip assay. So, the capacity of streptavidin- and NHS-modified biochips to detect the presence of TNFα (**Fig. 6A,B**) and TNFα antibody (**Fig. 6C,D**) was further investigated, respectively, in 51 EDTA-treated blood samples from 37 HLH patients. The results showed that there were reasonable concordances between streptavidin- and NHS-modified biochips in sorting of the infectious plasmas. The correlation coefficients (*r*) of TNFα and TNFα antibody assays between the 2 biochip formats were 0.9623 (**Fig. 7A**) and 0.9386 (**Fig. 7B**). Some of the positive samples by the streptavidin biochip assay appeared negative by the NHS biochip. The concordance frequencies in detecting TNFα and TNFα antibody were 92.2% (48/51) and 96.1% 49/51), respectively.

Blocking assays

The realities of biochip assay in detecting TNFα and TNFα antibody were checked. Positive TNFα and TNFα antibody reactions from 4 individual HLH plasmas were completely blocked even when pretreated by 0.05 μg/mL of standard TNFα antibody (**Fig. 8A**) and 0.1 μg/mL of TNFα antigen (**Fig. 8B**), respectively, before assaying.

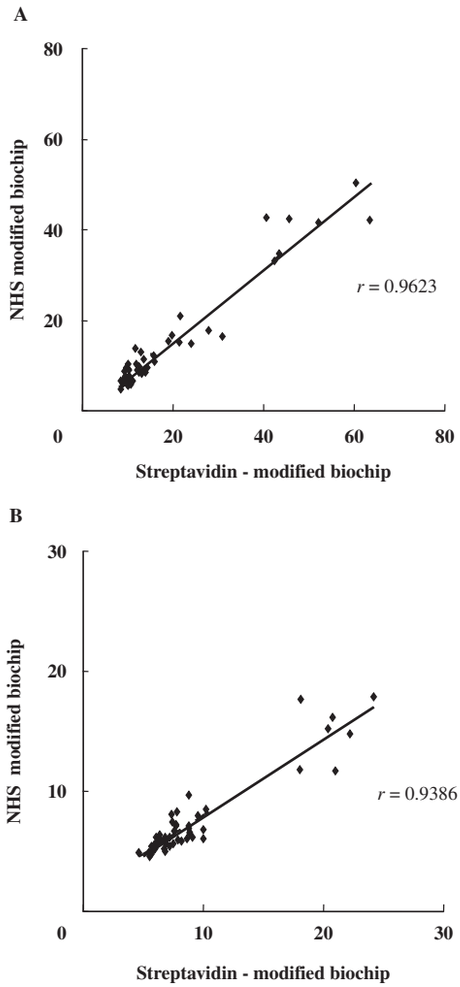


FIG. 7. The correlation coefficients (*r*) of (**A**) tumor necrosis factor–α (TNFα) and (**B**) TNFα antibody assays between the 2 biochip formats in detecting 51 samples from 37 hemophagocytic lymphohistiocytosis (HLH) patients.

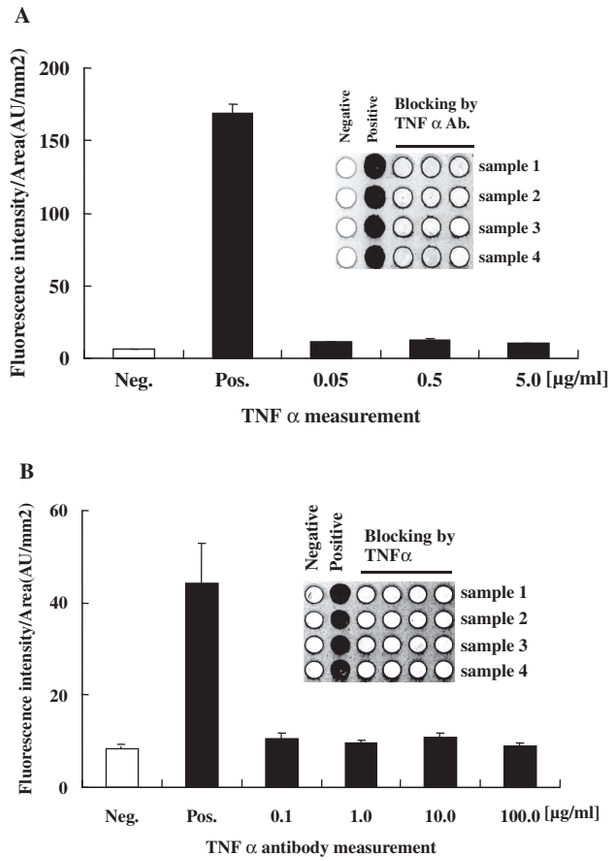


FIG. 8. Blocking assays of tumor necrosis factor–α (TNFα) and TNFα antibody reactions. Positive reactions of TNFα and TNFα antibody from 4 individual hemophagocytic lymphohistiocytosis (HLH) plasmas were completely blocked, even when pretreated by 0.05 μg/mL of (**A**) standard TNFα antibody and (**B**) 0.1 μg/mL of TNFα antigen, respectively.

Monitoring of TNFα responses in HLH patients by streptavidin-modified biochips

Plasma TNFα and the antibody were detected by the streptavidin biochip in the 71 HLH patients individually. Frequencies of plasma TNFα and TNFα antibody were 36.1% (26/71) and 22.2% (16/71), respectively. Meanwhile, plasma TNFα-TNFR1 and R2 complexes in 42 of 71 cases were measured. Frequencies of plasma TNFα, TNFα antibody, and TNFα-TNFR1 as well as R2 complexes in the 42 cases were 40.5% (17/42), 30.9% (13/42), 28.6% (12/42), and 26.1% (11/42), respectively. Almost all the HLH cases with a negative TNFα response did present the negative assays for TNFα antibody and the corresponding receptors. Only 1 case showed a positive TNFR1 reaction alone in the absence of TNFα and TNFα antibody responses. However, once TNFα antibody reaction was positive, a positive TNFα assay was expected (**Table 2**).

Finally, a longitudinal analysis (16 months) detecting TNFα, TNFα antibody, and its corresponding receptor (TNFR1 and 2) complexes in 18 plasma samples of a single patient during medication course of chemotherapy and following Zenapax (CD25 specific antibody) was performed. The results showed that after the application of CD25-specific antibody, the plasma levels of TNFα-relevant molecules decreased dramatically and close to the baseline (**Fig. 9A**) when compared with his sibling control (**Fig. 9B**).

DISCUSSION

In this study, we demonstrated that streptavidin-modified biochips produced the fluorescence intensity approximately 2 times higher than those of NHS-modified biochips. Both biochips produced various reproducibilities. The variability for

Table 2. Monitoring of TNFα Responses in 42 HLH Patients by Streptavidin-Modified Biochips

TNFα Biochip Assay	*Cases*
Ag+	2
Ab+	0
R1+	1
R2+	0
Ag+/Ab+	3
Ag+/R1+	1
Ag+/R2+	0
Ab+/R1+	0
Ab+/R2+	0
Ag+/Ab+/R1+	0
Ag+/Ab+/R2+	1
Ag+/Ab+/R1+/R2+	9
Ag+/Ab–/R1+/R2+	1
Ag–/Ab–/R1–/R2–	24
Total	42

Numbers in the table mean the positive cases. Ag: TNFα; Ab: TNFα antibody; R1: TNF receptor 1; R2: TNF receptor 2. TNFα, tumor necrosis factor–α; HLH, hemophagocytic lymphohistiocytosis.

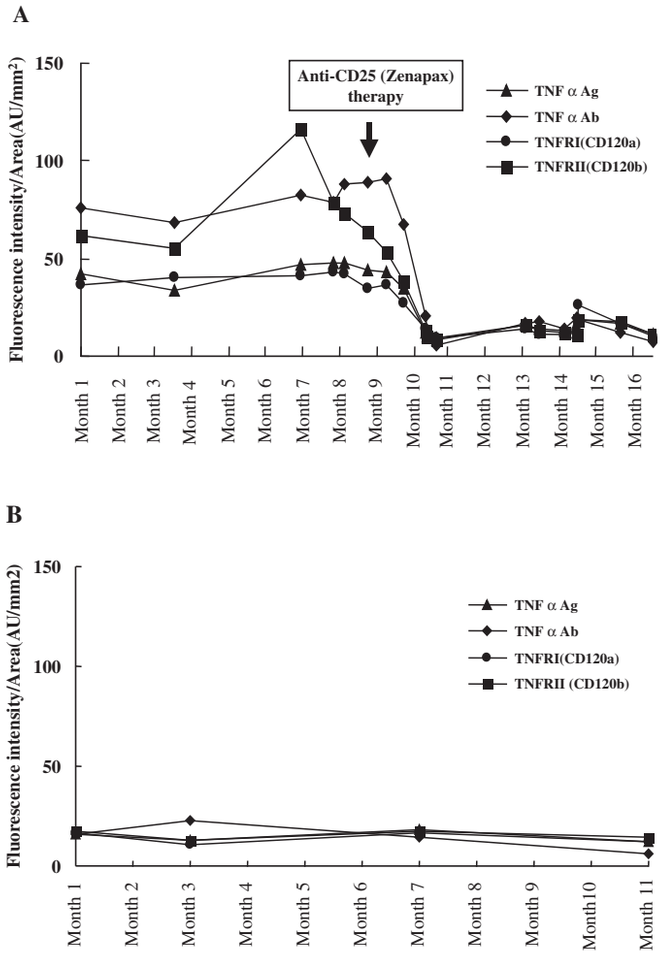


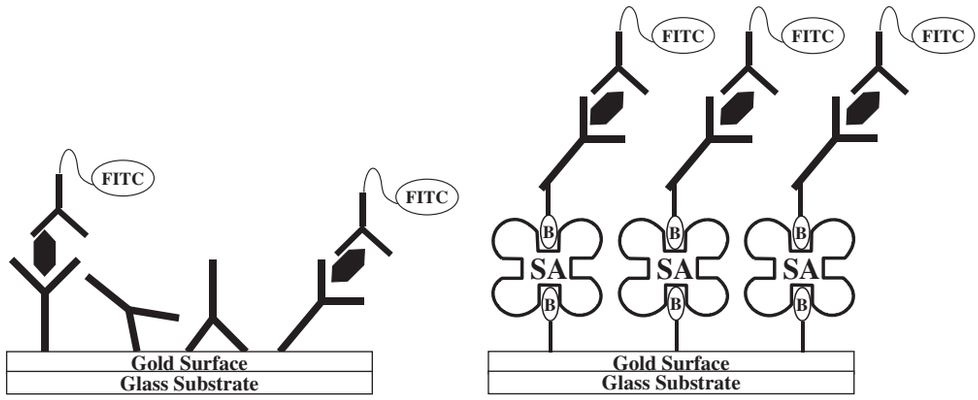
FIG. 9. Longitudinal analysis of tumor necrosis factor–α (TNFα), TNFα antibody, and its corresponding receptor (TNFR1 and 2) complexes in 18 plasma samples of a (**A**) single patient under chemotherapy and following anti-CD25 (Zenapax) therapy and (**B**) the patient's sibling. The arrow indicates the time when anti-CD25 therapy was started.

both biochips had acceptable coefficients of variation, varying from 1.87% to 4.56% on streptavidin-modified biochips and 5.03% to 8.64% on NHS-modified biochips. Clearly, fluorescence signals produced on streptavidin-modified biochips were more homogeneous and reproducible.

Blood preparation methods have variable effects on specific proteins or antibodies.[33] Anticoagulants may in some cases specifically interact with certain proteins or specifically affect the stability of certain proteins. Therefore, the development of assays for individual proteins needs to be evaluated and optimized on a case-by-case basis. In this study, the fluorescence intensities of TNFα and TNFα antibody in 7 pairs of plasma samples treated by either EDTA or heparin from 6 patients and 1 healthy individual were compared on streptavidin-modified biochips. EDTA-treated plasmas preserved more native activities of TNFα and TNFα antibody than heparin-treated ones. The reason why heparin attenuates plasma TNFα and TNFα antibody activity remains unclear. Comprehensive data regarding the suitability of different anticoagulants (EDTA, heparin, and sodium citrate) for plasma protein assays are quite controversial.[33-36] One group described that potential induction of TNFα in monocytes was more efficient in heparinized blood.[35] However, in contrast, the other highlighted isolated peripheral blood mononuclear cells (PBMCs) from EDTA anticoagulated blood showed a higher cytokine expression capacity than PBMCs from heparinized blood.[34] The levels of the cytokines TNFα, IL-6, and leptin were found to be highly variable in citrate- and heparin-treated plasma but not in EDTA-treated plasma or serum.[37] The relatively higher concentration of the cytokines in EDTA-plasma could indicate a protective effect of EDTA on cytokine stability, perhaps through EDTA's role as a protease inhibitor.[33] Other sources of variation in cytokine concentrations could be attributable to the interference in certain assays by anticoagulants or variability in protease activity or protein stability.[33]

Reasonable concordances between streptavidin- and NHS-modified biochips were found in measuring TNFα and TNFα antibody in 51 EDTA-treated plasma samples from 37 HLH patients. The correlation coefficients (*r*) by the 2 biochip formats were 0.9623 in the TNFα assay and 0.9386 in the TNFα antibody assay. Some of the positive samples by the streptavidin biochip assay appeared negative by the NHS biochip assay. The concordance frequencies of TNFα and TNFα antibody assays by the 2 biochip formats were 92.2% (48/51) and 96.1% (49/51), respectively. Obviously, the streptavidin-modified biochip created a more sensitive immunoassay than the NHS surface.

Many reasons led to variations in assaying serological proteins on biochip surfaces, presenting considerable heterogeneities



A. potentially random orientation on NHS surface **B. uniform orientation on streptavidin surface**

FIG. 10. Potential mechanism of assay heterogeneity from the 2 biochip surfaces in this study. FITC, fluorescein; NHS, N-hydroxysuccinimide.

among different biochip surface modifications. Protein binding might decrease due to nonuniform kinetic and thermodynamic properties when immobilized on the biochip surface.[3,24] In addition, orientation direction of immobilized proteins affects the production of specific fluorescence signals. In this study, a direct assessment for surface-immobilized protein was tested. Two strategies loading proteins onto biochip surfaces were used. One was to orient proteins randomly on the biochip surface via their intrinsic amine groups. The other was to immobilize a mixture of a biotin-terminated alkanethiol on gold in a certain ratio, which helps to optimize the binding density and reduces nonspecific binding to the surface to obtain an optimal packing condition for streptavidin. The streptavidin surface presents an ordered orientation of molecules on the biochip to expose valid antigen epitopes or recognition sites of probes to serum antibodies, thereby increasing biochip assay sensitivity reasonably.[1,10,21] Theoretically, NHS active ester shows a high reactivity and selectivity toward primary amino groups; thereby, any proteins with nucleophilic groups (e.g., amino groups of lysine residuals of protein/enzyme molecules) would result in stable binding to NHS ester on microarrays.[20] However, the arrangement of NH_2-containing proteins on the NHS-modified biochip surface was stochastic because the binding alignment of proteins to the surface was at random. The spontaneous binding of NH2 side chains from the proteins to the NHS surface probably altered the epitope structure and/or the orientation of a proportion of the antigen, leading to a partial recognition exposure or complete blockade of protein and protein interaction (**Fig. 10**). Thus, the access for proteins to be detected to the surface might be impaired. The heterogeneity for surface-immobilized proteins would be related not only to steric effects, protein conformational changes, and even denaturation but also to orientation of protein molecules immobilized. Therefore, in this study, although both the biochips shared a similar specificity in assaying human serological TNFα and its relevant proteins, streptavidin-modified biochips are more sensitive than NHS-modified biochips.

The shelf life period of protein biochip products was considerably affected by protein degradation or protein conformational changes on biochips. For preserving proteins on biochip surface active, many potential protective substances were tested as stabilizers in protein shelf life and immunoassays, such as glycerol,[11,21] disaccharides,[29] saccharose,[38] low molecular polyethylene glycol (PEG),[39] and PEO.[40] In this study, it is evident that TNFα antigen- and antibody-coated streptavidin and NHS-modified biochips on gold, when preserved at 4 °C under desiccated conditions, were quite stable without any significant attenuation effect on fluorescence signal intensity. The detection of fluorescence signals was still acceptable even at week 8.

HLH is a rare disease occurring either as a sporadic or familial form in newborns and young children.[24-26,41] The disease initially presents as a severe sepsis-like illness with massive hepatosplenomegaly, inflammation, and variable neurological symptoms in addition to anemia and other cytopenias. Neonatal sepsis is often diagnosed, but the disease is resistant to antibiotic treatment and may be rapidly fatal if not treated specifically. TNFα may induce apoptosis and cell injuries via binding to TNFR1 to activate the TRAF2/TRADD/FADD (TNFR-associated factor 2/TNFR1-associated death domain/Fas-associated DD) signaling and caspase activities.[42,43] So far, this context has not yet been addressed in patients with injury-associated hyperinflammation, prolonged sepsis, and chronic infections. Furthermore, whether TNF-specific autoantibodies play a role in hyperinflammatory syndromes apparently resistant to autochthonous anti-inflammatory responses remains unknown. In this study, the plasma levels of TNFα, TNFα antibody, and TNFα-receptor complexes (TNFR1 and 2) increased in HLH cases. Frequencies of the markers were 40.5% (17/42), 30.9% (13/42), 28.6% (12/42), and 26.1% (11/42), respectively. Appearances of TNFα antibody indicated

that an autoimmune mechanism might be involved in HLH pathogenesis at least in some of the patients and may partially explain why TNFα plasma concentrations appear to be less elevated than other inflammatory cytokines. Using an indirect immunoassay strategy, complexes of TNFR1 and 2 bound to TNFα in the plasmas were successfully detected. The data suggested that the soluble TNF receptors binding to TNF might play a regulatory role in the modulation of the inflammatory response and feedback inhibition.[42,44] This finding provides a potential mechanism to explain the obstinate disease persistence or poor prognosis for HLH patients.

Finally, longitudinal biochip analysis (16 months) investigating plasma TNFα response of a single HLH patient under chemotherapy and following Zenapax (CD25-specific antibody) therapy was performed. The results showed that after the application of CD25-specific antibody, the plasma levels of TNFα-relevant molecules decreased dramatically and were close to baseline levels when compared with the sibling control. Zenapax® (daclizumab) is an immunosuppressive, humanized IgG1 monoclonal antibody produced by recombinant DNA technology that binds specifically to the α subunit (p55 alpha, CD25, or Tac subunit) of the human high-affinity IL-2 receptor that is expressed on the surface of activated lymphocytes. However, the effect of Zenapax on eliminating TNFα in the treatment of HLH still remains unclear, although the rationale for its use is strong.[45] It was supposed that Zenapax medication that can bind to CD25 might be able to decrease the severity of HLH by eliminating the activity of CD25 and depressing all the effective cells involved in HLH and thereby indirectly affecting the production of TNFα.

In conclusion, the potential application of this protein biochip format can be extended to rapidly and reliably test the plasma molecules for not only HLH but also other autoimmune diseases. Similar to Western blot, the indirect immunoassay strategy on the biochip for detecting plasma TNFα-TNFR1 or 2 complexes offers a potential approach for capturing any immunocompetent and/or integrated molecules in sera and cell lysates.

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