

RESEARCH ARTICLE

Specific activation of pro-Infliximab enhances selectivity and safety of rheumatoid arthritis therapy

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Abstract

During rheumatoid arthritis (RA) treatment, long-term injection of antitumor necrosis factor α antibodies (anti-TNF α Abs) may induce on-target toxicities, including severe infections (tuberculosis [TB] or septic arthritis) and malignancy. Here, we used an immunoglobulin G1 (IgG1) hinge as an Ab lock to cover the TNF α -binding site of Infliximab by linking it with matrix metalloproteinase (MMP)-2/9 substrate to generate pro-Infliximab that can be specifically activated in the RA region to enhance the selectivity and safety of treatment. The Ab lock significantly inhibits the TNF α binding and reduces the anti-idiotypic (anti-Id) Ab binding to pro-Infliximab by 395-fold, 108-fold compared with Infliximab, respectively, and MMP-2/9 can completely restore the TNF α neutralizing ability of pro-Infliximab to block TNF α downstream signaling. Pro-Infliximab was only selectively activated in the disease site (mouse paws) and presented similar pharmacokinetics (PKs) and bio-distribution to Infliximab. Furthermore, pro-Infliximab not only provided equivalent therapeutic efficacy to Infliximab but also maintained mouse immunity against *Listeria* infection in the RA mouse model, leading to a significantly higher survival rate (71%) than that of the Infliximab treatment group (0%). The high-selectivity pro-Infliximab maintains host immunity and keeps the original therapeutic efficiency, providing a novel strategy for RA therapy.

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Abbreviations: %ID/g, percent injected dose per gram; Ab, antibody; ADA, antidrug antibody; anti-Id, anti-idiotypic; AUC, area under the plasma concentration; CDR, complementarity-determining region; CFU, colony-forming unit; Cl, clearance value; C_{max} , mean maximum serum concentration; DBA/1J, dilute brown non agouti; EC_{50} , half-maximal effective concentration; FDA, Food and Drug Administration; HBsAg, HBV surface antigen; HBV, hepatitis B; HE, hematoxylin-eosin; IgG1, immunoglobulin G1; IL-6R, interleukin-6 receptor; LAP, latency-associated peptide; MMP, matrix metalloproteinase; MRT, mean residence time; MW, molecular weight; NF- κ B, nuclear factor kappa B; PK, pharmacokinetic; RA, rheumatoid arthritis; TB, tuberculosis; TCZ, Tocilizumab; Tg197 mice, hTNF α -transgenic mice; TNF α , tumor necrosis factor α ; TNFKO, TNF α knockout.

Introduction

Antitumor necrosis factor α antibodies (anti-TNF α Abs) constitute a major advance in rheumatoid arthritis (RA) therapy in the clinic, as targeting TNF α in the disease region can reduce pathological inflammation and efficiently inhibit RA progression [1]. In addition to their biological importance, anti-TNF biologics also constitute the most profitable drug class in history, exceeding US\$25 billion total sales globally [2]. However, TNF α is not only a critical proinflammation factor for RA patients but also a key immune modulator of host defense and tumor growth control [3]. The United States Food and Drug Administration (FDA) has reported that systemic injection of anti-TNF α Abs (Infliximab [4] and Adalimumab [5]) can lead to serious adverse effects (on-target toxicities) such as severe infections [6, 7], reactivation of viral infections (hepatitis or herpes zoster) [8, 9], and elevated risk of malignancy [4, 5, 10]. Singh and colleagues indicated the risk of serious infections is increased 30% during the treatment of anti-TNF α therapy [11], and common infections such as tuberculosis (TB), bacterial sepsis, *Streptococcus pneumoniae*, and *Listeria monocytogenes* might lead to hospitalization or death. In addition, Viganò and colleagues reported the reactivation of hepatitis B (HBV), leading to severe rheumatic complications in 23 HBV surface antigen (HBsAg)-positive patients treated with TNF α inhibitors, leading to nine cases of fulminant hepatitis, four deaths, and one liver transplantation [9]. Moreover, Mariette and colleagues suggested that the anti-TNF α Abs increase the risk of lymphoma 2- to 3-fold compared with the general population [12]. Thus, there is an urgent need to solve the on-target toxicities that accompany anti-TNF α therapy to provide higher selectivity and a safe treatment for RA patients.

Several strategies have been used in the clinic to prevent the on-target toxicities, which are induced by anti-TNF α therapies. For example, vaccination can prevent the risk of pneumonia and HBV; however, not all infections can be prevented by vaccine (e.g., listeriosis) [13]. Further, the systemic immunosuppression of anti-TNF α therapies hampers the immunogenicity of these vaccines, and in addition, live vaccine (i.e., herpes zoster) was contraindicated in anti-TNF α -treated patients, as it may cause infection from the vaccine virus strain. Therefore, immunosuppressant medication limits the application of vaccinations in RA patients. In addition, screening for latent TB, HBV, and cancer before initiation of anti-TNF α therapies has been recommended to perform safer treatment. Nevertheless, 10% of RA patients still suffer from new TB infection [14]; 12.5% of inactive HBsAg carriers with arthritis showed HBV reactivation due to low or undetectable HBV [15]. Systemic immunosuppression associated with anti-TNF α therapies is widely believed to be a confounding factor in infection and HBV reactivation development. On the other hand, to prevent the risk and recurrence of lymphoma associated with anti-TNF α Abs for RA patients, rituximab (anti-CD20 Ab, Mabthera) was recommended to replace anti-TNF α Abs, which can be a dual agent that inhibits the progression of lymphoma and RA disease activity [16]. However, the systemic injection of rituximab also caused on-target toxicities, including fatal infusion and progressive multifocal leukoencephalopathy [17]. Therefore, it is desirable to develop an anti-TNF α Ab that is highly selective to the RA region to enhance safety for use in RA.

In this study, we developed an “antibody lock” (Ab lock) that provides Infliximab with disease selectivity. We used the autologous hinge region as an Ab lock to block the antigen-binding site of Infliximab through matrix metalloproteinase (MMP)-2/9 substrate as a linker to generate pro-Infliximab. After the Ab lock is removed by expression of MMP-2/9 in the disease region, the cleaved pro-Infliximab is expected to be specifically activated and neutralize the target antigen to suppress RA progression (Fig 1). We first examined the masking efficiency of the Ab lock on pro-Infliximab and then confirmed whether MMP-2/9 treatment

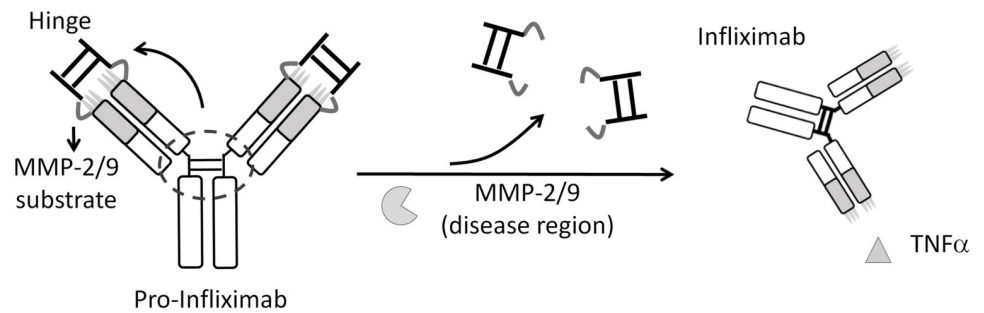


Fig 1. An autologous hinge as an Ab lock to enhance the selectivity and maintain the host immunity of Infliximab. We used an autologous IgG1 hinge as Ab lock to cover the antigen-binding site of Infliximab by using MMP-2/9 substrate linker to generate pro-Infliximab. Upon protease activation at the RA region, the Ab lock was released, and the pro-Infliximab could specifically activate and neutralize TNF α at the disease site to inhibit RA progression. Ab, antibody; IgG1, immunoglobulin G1; MMP, matrix metalloproteinase; RA, rheumatoid arthritis; TNF α , tumor necrosis factor α .

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could completely restore the binding ability of the pro-Infliximab to block the TNF α downstream signaling. Next, we tested whether the Ab lock could prevent the binding of anti-idiotypic (anti-Id) Ab to pro-Infliximab by immunoassay. We compared the biodistribution, serum half-life, MMP-2/9-dependent selective activation, and therapeutic efficacy of pro-Infliximab with Infliximab in mice. Finally, we tested whether pro-Infliximab reduces the risk of opportunistic infection and extended adverse events in an RA mouse model challenged with *Listeria*, using Infliximab as a control. The successful development of a highly selective pro-Infliximab potentially provides a safer option to prevent the systemic on-target toxicities associated with anti-TNF α therapies and improve the quality of life of patients.

Results

Ab lock can reversibly mask the TNF α -neutralizing ability of Infliximab

We first evaluated the masking effect of the Ab lock by comparing the TNF α -binding ability of Infliximab and the pro-Infliximab by ELISA. The half-maximal effective concentration (EC₅₀) of Infliximab and pro-Infliximab were 0.78 ± 0.05 nM and 308.60 ± 13.88 nM, respectively, showing that pro-Infliximab has an approximately 395-fold weaker antigen-binding ability than Infliximab (Fig 2A). Then, we investigated whether the Ab lock can be efficiently removed from pro-Infliximab and restore the TNF α -binding ability of pro-Infliximab after MMP-2/9 cleavage by western blot and ELISA. The results demonstrated that MMP-2/9 could completely remove the Ab lock from pro-Infliximab (S1 Fig), and the TNF α -binding ability of pro-Infliximab was gradually elevated in a time-dependent manner during MMP-2/9 treatment (S2 Fig). Further, after reacting with MMP-2/9 protease, the EC₅₀ of pro-Infliximab was elevated from 308.60 ± 13.88 nM to 1.15 ± 0.18 nM and showed no significant difference ($P = 0.89$) as compared with control Infliximab. These results indicate that the antigen-binding ability of pro-Infliximab could be completely restored by MMP-2/9 cleavage. We next assessed the TNF α -neutralizing ability of MMP-2/9-activated pro-Infliximab by nuclear factor kappa B (NF- κ B)-responsive reporter assay [18]. Fig 2B shows that the luciferase activity of pro-Infliximab-treated cells was similar to the TNF α treatment group. In contrast, MMP-2/9-activated pro-Infliximab significantly neutralized TNF α and reduced the luciferase activity (at least 283.3-fold as compared with inactive pro-Infliximab group) similar to that of the Infliximab-treated group ($P > 0.05$), suggesting that the MMP-2/9-activated pro-Infliximab could efficiently neutralize TNF α and block the downstream NF- κ B signaling. Together, these results

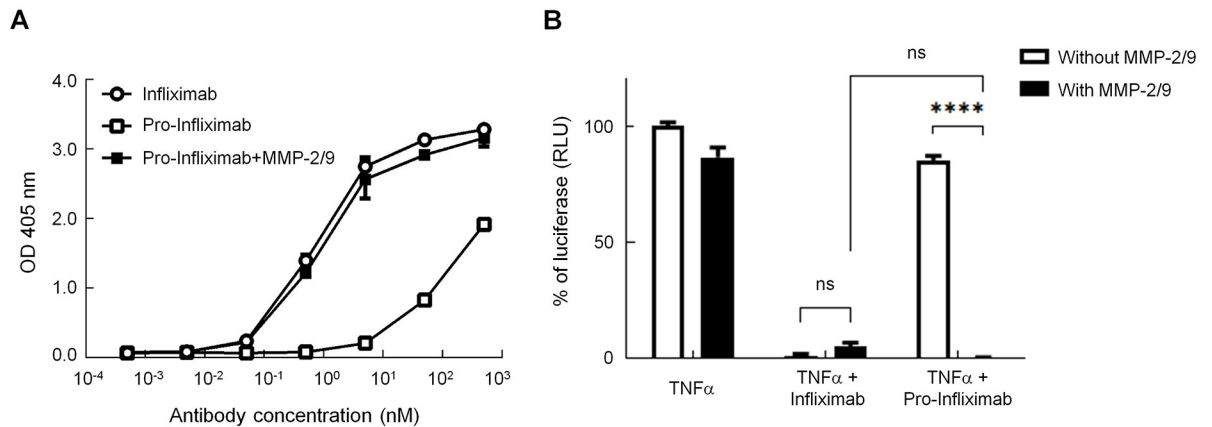


Fig 2. The Ab lock inhibits the binding ability and TNF α downstream signaling of Infiximab. (A) The binding ability of Infiximab (○), pro-Infiximab (□), and MMP-2/9-activated pro-Infiximab (■) was assessed by TNF α -based ELISA. (B) TNF α -neutralizing ability of pro-Infiximab and Infiximab with or without MMP-2/9 through luciferase NF- κ B reporter assay. TNF α treatment was used as a control for reporter activity. The values are mean \pm SEM, and the asterisks indicate a significant difference, **** P < 0.0001. Error bar: standard error of triplicate determinations. Underlying data can be found in [S1 Data](#). Ab, antibody; MMP, matrix metalloproteinase; NF- κ B, nuclear factor kappa B; NS, no significance; TNF α , tumor necrosis factor α .

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indicate that the Ab lock markedly masks the antigen-recognition ability of Infiximab, and inhibition of the biological function of pro-Infiximab could be completely restored after MMP-2/9 protease treatment.

The Ab lock prevents the neutralizing effect of an anti-Id Ab on Infiximab

Previous studies have reported that nearly half RA patients develop anti-Infiximab Abs, which are mostly known as anti-Id Abs, within the first year of Infiximab treatment [19, 20]. A high level of anti-Id Abs decreases the bioavailability and serum half-life of Infiximab, thereby impairing its therapeutic efficacy in RA patients [3, 21]. To evaluate whether the spatial-hindrance-based Ab lock could prevent pro-Infiximab bound by anti-Id Abs of Infiximab (anti-I-Id Abs), we immobilized the anti-I-Id Abs (Bio-Rad Laboratories, Redmond, WA, USA) on a plate and analyzed the binding ability of pro-Infiximab and Infiximab in the condition with or without MMP-2/9 by ELISA. [Fig 3A](#) indicates that the anti-I-Id Ab binding ability of pro-Infiximab (EC_{50} = 72.93 nM) was significantly lower than Infiximab (EC_{50} = 0.67 nM) and it can be completely restored after MMP-2/9 treatment, suggesting that pro-Infiximab possess approximately 108-fold weaker anti-I-Id Ab binding activity as compared to control Infiximab and the neutralizing effect of anti-I-Id Ab is reversible. To further investigate the effect of anti-I-Id Abs on the restoration ability of TNF α binding of pro-Infiximab by protease, we immobilized pro-Infiximab and Infiximab in plates and then treated them with different concentrations of anti-I-Id Abs. After removing unbound anti-I-Id Abs, we incubated the samples with or without MMP-2/9 and examined the TNF α binding ability by TNF α -biotin and horseradish peroxidase (HRP)-conjugated streptavidin. As shown in [Fig 3B](#) the TNF α binding ability of Infiximab and MMP-2/9-treated Infiximab were gradually decreased in a dose-dependent manner of anti-I-Id Abs. In contrast, the antigen binding ability of pro-Infiximab could be completely restored by treatment with MMP-2/9 and was comparable to control Infiximab without the presence of anti-I-Id Abs. These results suggest that the Ab lock was able to prevent the neutralizing effect of the anti-Id Abs of Infiximab whilst maintaining the restoration ability of pro-Infiximab by MMP-2/9.

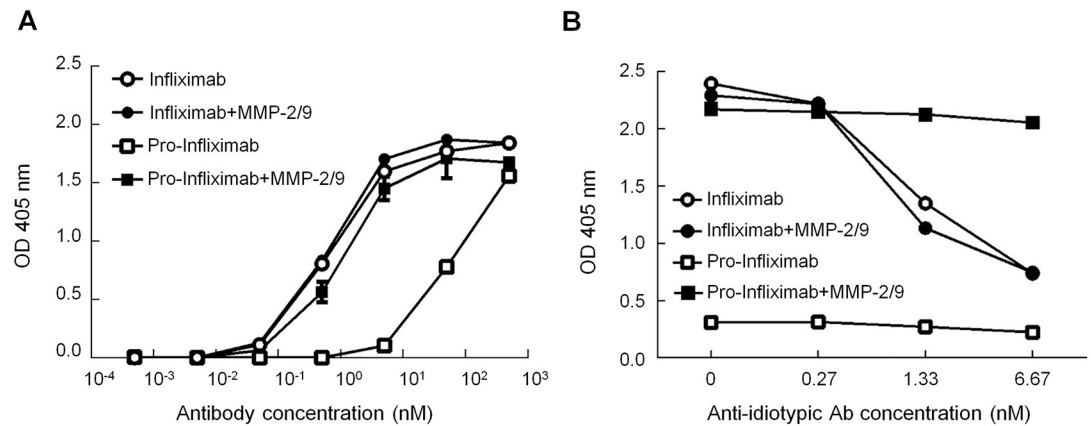


Fig 3. The Ab lock can prevent the response of the anti-Id Ab to Infliximab. (A) The anti-I-Id Ab-coated 96-well plate was incubated with different concentrations of Infliximab-biotin (○), pro-Infliximab-biotin (□), Infliximab-biotin preincubated with MMP-2/9 (●), or pro-Infliximab-biotin preincubated with MMP-2/9 (■), respectively, and then analyzed the neutralizing ability of anti-I-Id Ab by ELISA. (B) To further investigate the effect of anti-I-Id Abs on the restoration ability of TNF α binding of pro-Infliximab by protease. The Infliximab- or pro-Infliximab-coated plates were treated with different concentrations of anti-I-Id Abs. After removing unbound anti-I-Id Abs, we incubated the samples with or without MMP-2/9 and examined the TNF α -binding ability by TNF α -biotin and HRP-conjugated streptavidin. The values are mean \pm SEM. Error bar: standard error of triplicate determinations. Underlying data can be found in [S1 Data](#). Ab, antibody; anti-I-Id, anti-Infliximab idiotypic; HRP, horseradish peroxidase; MMP, matrix metalloproteinase; TNF α , tumor necrosis factor α .

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The Ab lock does not change the basic characteristics of Infliximab

To determine whether the Ab lock altered the basic properties of Infliximab, including the serum half-life and biodistribution, dilute brown non agouti (DBA/1J) mice were intraperitoneally injected with ¹³¹I-labeled pro-Infliximab or ¹³¹I-labeled Infliximab. The blood and organ specimens were collected, and the radioactivity was measured by a multichannel gamma counter at different time points after administration of radio-labeled Abs. As shown in [Fig 4A](#), the average radioactivity concentrations of both pro-Infliximab and Infliximab in blood samples were gradually decreased and showed a similar curve profile post injection of Abs. The serum half-life of pro-Infliximab and Infliximab were 84.14 h and 83.98 h ([Table 1](#)). The pharmacokinetic (PK) analysis also demonstrated the exposure to pro-Infliximab as measured by the mean maximum serum concentration (C_{max}), and the area under the plasma concentration (AUC) was similar to those of Infliximab. Additionally, both of the antibodies displayed lower clearance values (CI) and longer mean residence time (MRT) without significant differences. These results demonstrated that the Ab lock did not alter the PK characteristics of pro-Infliximab in circulation. Regarding biodistribution, we also observed that there was no significant difference (*P* > 0.05) in radioactivity (percent injected dose per gram [%ID/g]) between each corresponding organ derived from the pro-Infliximab- and Infliximab-injected mice at 12, 24, 96, and 168 h ([Fig 4B–4E](#)). These results suggest that the Ab lock did not affect the basic biological properties of pro-Infliximab and maintained PK and biodistribution similar to the control, Infliximab, in vivo.

Site-specific activation of pro-Infliximab prevents RA progression

To examine whether pro-Infliximab could be selectively activated in the RA region, hTNF α -transgenic 1006 mice, which can spontaneously develop moderate arthritis in the hind paws, were treated with pro-Infliximab or Infliximab. Blood and hind paw specimens were collected at different time points, and the molecular weight profile of pro-Infliximab and Infliximab were evaluated by western blot. Here, we used light chain molecular weight as an indicator of

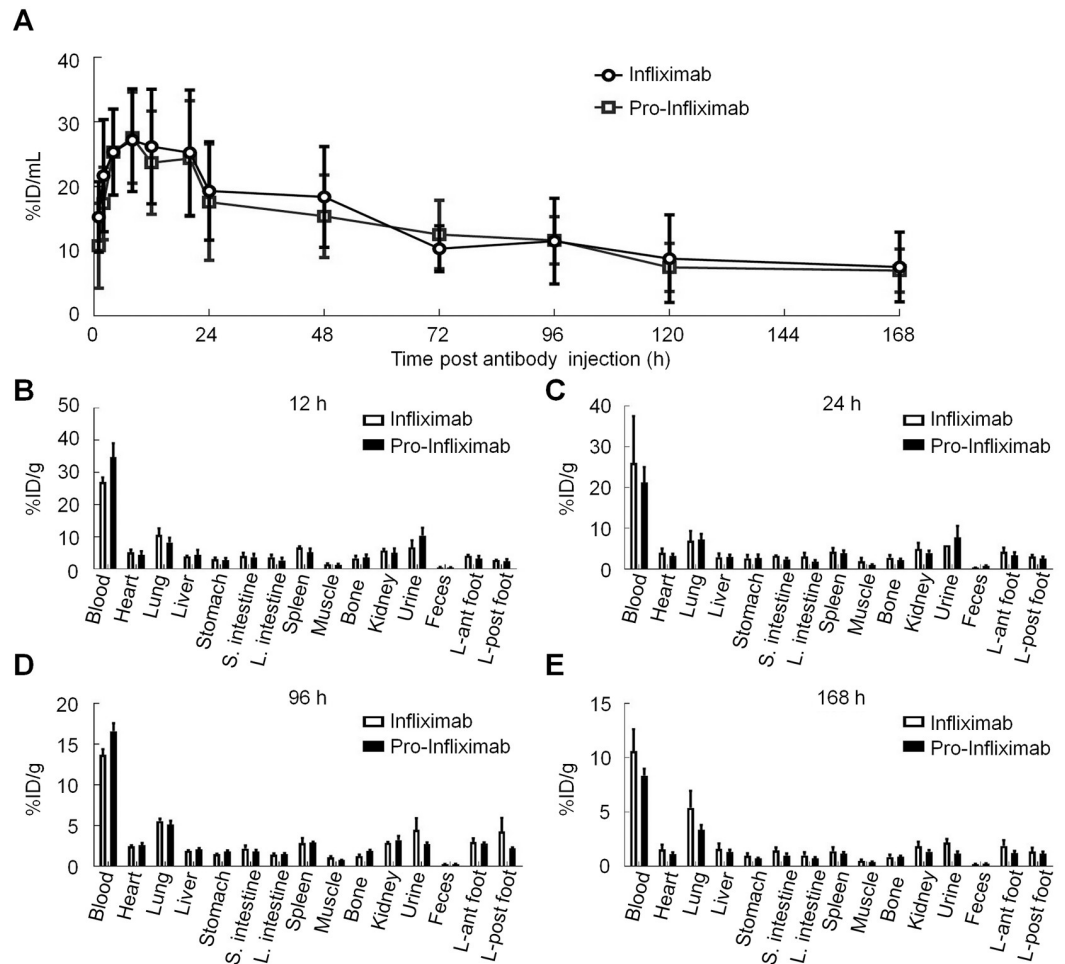


Fig 4. Evaluation of the PK properties and biodistribution of pro-Infliximab and Infliximab. The DBA/1J mice were intraperitoneally injected with ¹³¹I-labeled Infliximab (○) or ¹³¹I-labeled pro-Infliximab (□). The blood was collected at different time and the radioactivity was detected via the γ counter to measure the (A) PK properties of ¹³¹I-labeled Infliximab and ¹³¹I-labeled pro-Infliximab. The biodistribution of ¹³¹I-labeled Infliximab and ¹³¹I-labeled pro-Infliximab in several tissues and organs in DBA/1J mice at 12 h (B), 24 h (C), 96 h (D), and 168 h (E) after injection. PK properties and biodistribution are expressed as radioactivity %ID/g. Error bar: standard error of triplicate determinations. Underlying data can be found in S1 Data. DBA/1J, dilute brown non agouti; %ID/g, percent injected dose per gram; PK, pharmacokinetic.

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protease restoration. Fig 5A and 5B indicate that the light chain molecular weight (25.6 kDa) in the paw of Infliximab-treated mice was consistent with the molecular weight profile in the blood. By contrast, the light chain molecular weight (29.4 kDa) in the paws of pro-Infliximab-treated hTNF α -transgenic 1006 mice were only gradually converted into cleaved form

Table 1. The Ab lock does not change the PK characteristics of Infliximab.

Test article	C _{max} (%ID/mL)	AUC (h*%ID/mL)	CI (mL/h)	MRT (h)	Half-life (h)
Infliximab	27.2	2,280.1	0.03	128.9	84.0
Pro-Infliximab	27.6	2,147.5	0.03	128.3	84.1

Abbreviations: %ID/g, percent injected dose per gram; AUC, area under the plasma concentration; CI, clearance value; C_{max}, mean maximum serum concentration; h, hour; MRT, mean residence time.

Additionally, plasma CI and MRT are shown.

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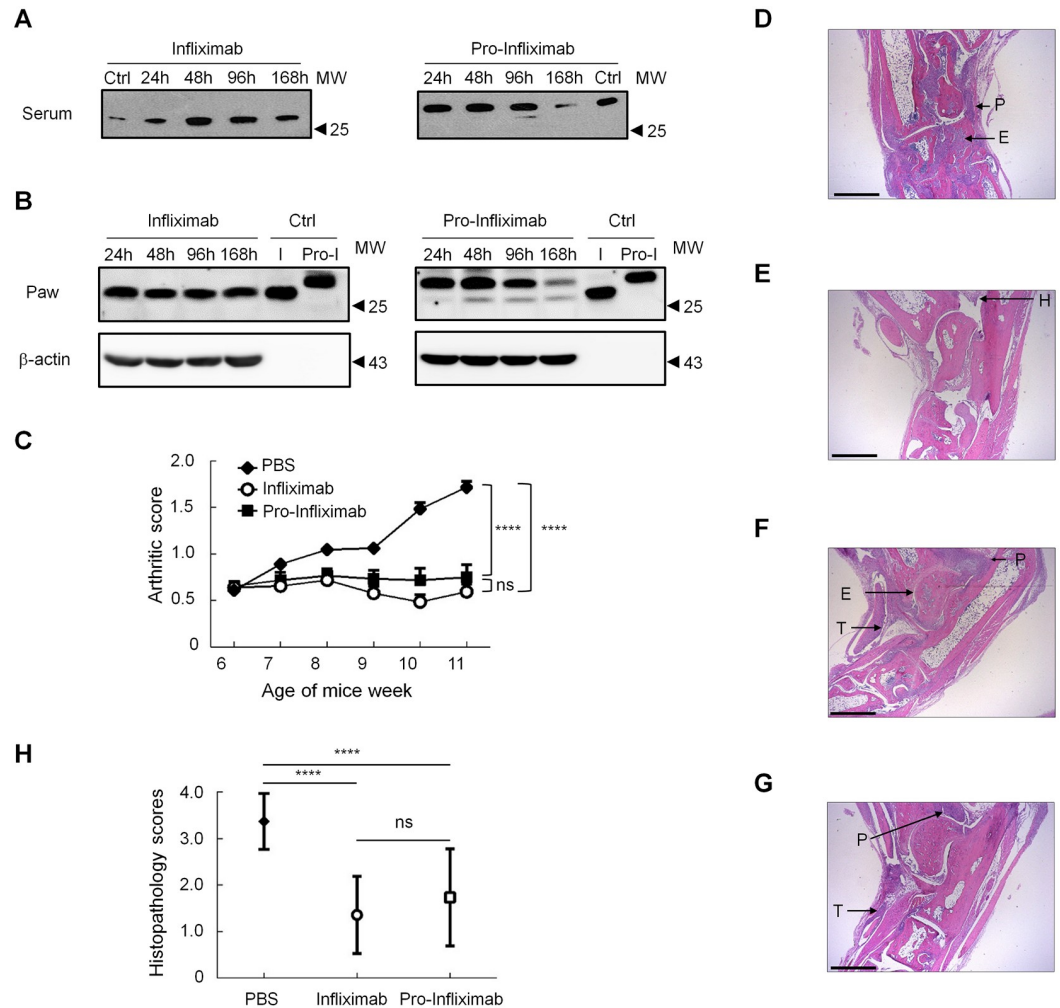


Fig 5. The pro-Infliximab is activated at the disease site and suppresses disease development. The hTNF α -transgenic 1006 mice were intraperitoneally injected with 50 μ g Infliximab or pro-Infliximab. After 24, 48, 96, and 168 h, the blood and hind paw specimens were collected and using HRP-conjugated antihuman IgG Fab Ab for detecting the level of active and inactive pro-Infliximab in (A) serum and (B) joint tissue (paw) by western blot. The β -actin as internal control. I means Infliximab, pro-I means pro-Infliximab. The Tg197 mice ($n = 8$) were intraperitoneally injected with 10 mg/kg Infliximab (\circ), 10 mg/kg pro-Infliximab (\blacksquare), or saline (\blacklozenge). All mice were dosed twice weekly for 6 weeks. (C) Arthritic score of each group was monitored every week to measure the therapeutic efficacy. Representative HE stained sagittal tissue sections of the ankle joints in Tg197 mice after treatment with PBS (D), Infliximab (E, F), and pro-Infliximab (G) for 6 weeks. Representative histopathology images at 25 \times magnification. Bars = 1 mm. (H) The histopathology score of the paw in PBS, Infliximab, and pro-Infliximab-treated Tg197 mice. The values are mean \pm SD, and the asterisks indicate a significant difference, * $P < 0.01$, ** $P < 0.001$. Error bar: standard error of octuplicate determinations. Underlying data can be found in [S1 Data](#). Ab, antibody; E, bone erosion; H, synovial hyperplasia; HE, hematoxylin-eosin; HRP, horseradish peroxidase; IgG; NS, no significance; P, pannus; T, tendonitis; Tg197 mice, hTNF α -transgenic mice; TNF α , tumor necrosis factor α .

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(25.6 kDa) at 24, 48, 96 and 168 h, but there was only a slightly cleaved form observed in the blood at 168 h (Fig 5A and 5B). Further, pro-Infliximab was not activated by protease in lung tissue of pro-Infliximab-treated mice. And both Infliximab and pro-Infliximab were gradually degraded in a time-dependent manner after Ab treatment. However, there were no Infliximab or pro-Infliximab signals detected in colon and spleen tissue (S3 Fig). These results indicated that the Ab lock could be selectively released from pro-Infliximab in the RA region. To further investigate the preventive effect of pro-Infliximab and Infliximab on pathological progression

of RA, hTNF α -transgenic mice (Tg197 mice), which can also spontaneously develop arthritis in the hind paws with deformed ankle joints at 4 weeks of age, were intraperitoneally injected with 10 mg/kg pro-Infliximab or Infliximab twice a week for 6 weeks by Biomedcode (Vari, Greece) animal facilities. The clinical score of pro-Infliximab- and Infliximab-treated Tg197 mice were significantly decreased as compared with control mice treated with normal saline ($P < 0.0001$, Fig 5C). Of note, there was no significant difference between the arthritis scores in either the pro-Infliximab- or Infliximab-treated groups (Fig 5C), suggesting that pro-Infliximab prevents RA in a similar manner to the control Infliximab. Both of these two Ab-treated mice gained more body weight than the normal saline-treated group (S4 Fig). In addition, the joint damage in hind paws was also evaluated by histopathologic analysis through hematoxylin-eosin (HE) staining. All joints were scored for synovitis, bone erosions, and cartilage degradation using a predefined scoring system [22]. Six weeks post-treatment, saline-treated Tg197 mice showed increased histopathologic scores for bone erosion, cartilage destruction, and pannus in hind paws (Fig 5D). In contrast, the synovial inflammation, bone erosions, and cartilage degradation were almost completely resolved in pro-Infliximab- and Infliximab-treated groups in comparison with the saline-treated group ($P < 0.0001$, Fig 5D–5G). Moreover, there was no significant difference in the histopathologic scores between the pro-Infliximab- and Infliximab-treated Tg197 mice ($P = 0.29$, Fig 5H). Collectively, these results indicate that pro-Infliximab could be specifically reactivated in the RA region and decreases RA progression with comparable efficacy to Infliximab.

Selective restoration of pro-Infliximab reduces risk of opportunistic infection

Previous studies and an FDA report mentioned that long-term administration of Infliximab increases susceptibility to serious infections, even leading to hospitalization or death of RA patients. Listeriosis, caused by *L. monocytogenes*, is one of the most commonly reported opportunistic infections, which may cause sepsis and meningitis, in Infliximab-treated RA patients [23, 24]. To investigate whether the regionally restored pro-Infliximab reduces the risk of opportunistic infection during Ab treatment, hTNF α -transgenic mice (Tg1278 mice), which only express normally regulated hTNF α but lack mTNF α [25], were intraperitoneally injected with 1 and 10 mg/kg of pro-Infliximab, Infliximab, or control saline before being challenged with a lethal dose of *L. monocytogenes* ($\times 10^4$ colony-forming unit [CFU]). Blood and liver tissue specimens were collected to analyze the CFUs of *L. monocytogenes* and the histopathologic phenotype, respectively. The number of *Listeria* CFUs in the blood from pro-Infliximab- or saline-treated mice was significantly lower ($****P < 0.0001$) than those from blood samples from Infliximab-treated mice and TNF α knockout (TNFKO) mice (Fig 6A), indicating that systemic TNF α is not recognized by pro-Infliximab and protects the host from *L. monocytogenes* infection. On the other hand, the histopathologic data of liver tissue from pro-Infliximab-treated mice exhibited fewer infectious foci (1–2 per microscope field) and infiltrated immune cells in comparison with the Infliximab-treated groups (4–5 per microscope field, Fig 6B). The overall survival rate of pro-Infliximab-treated mice was also significantly longer than Infliximab-treated Tg1278 mice and untreated TNFKO mice (mTNF α : $-/-$, hTNF α : $-/-$) in 1 and 10 mg/kg dosage administration (Fig 6C and 6D) and showed 71.4% and 37.5% survival until the end of the study, respectively. In contrast, mice treated with a low dose (1 mg/kg) or high dose (10 mg/kg) of Infliximab showed 100% lethality within 7 days similar to *Listeria*-challenged TNFKO mice, indicating that the systemic blockage of TNF α by Infliximab destroys the TNF-dependent host defense mechanism and aggravates infection of *Listeria* in the host. Together, these results suggest that pro-Infliximab may not affect systemic TNF α .

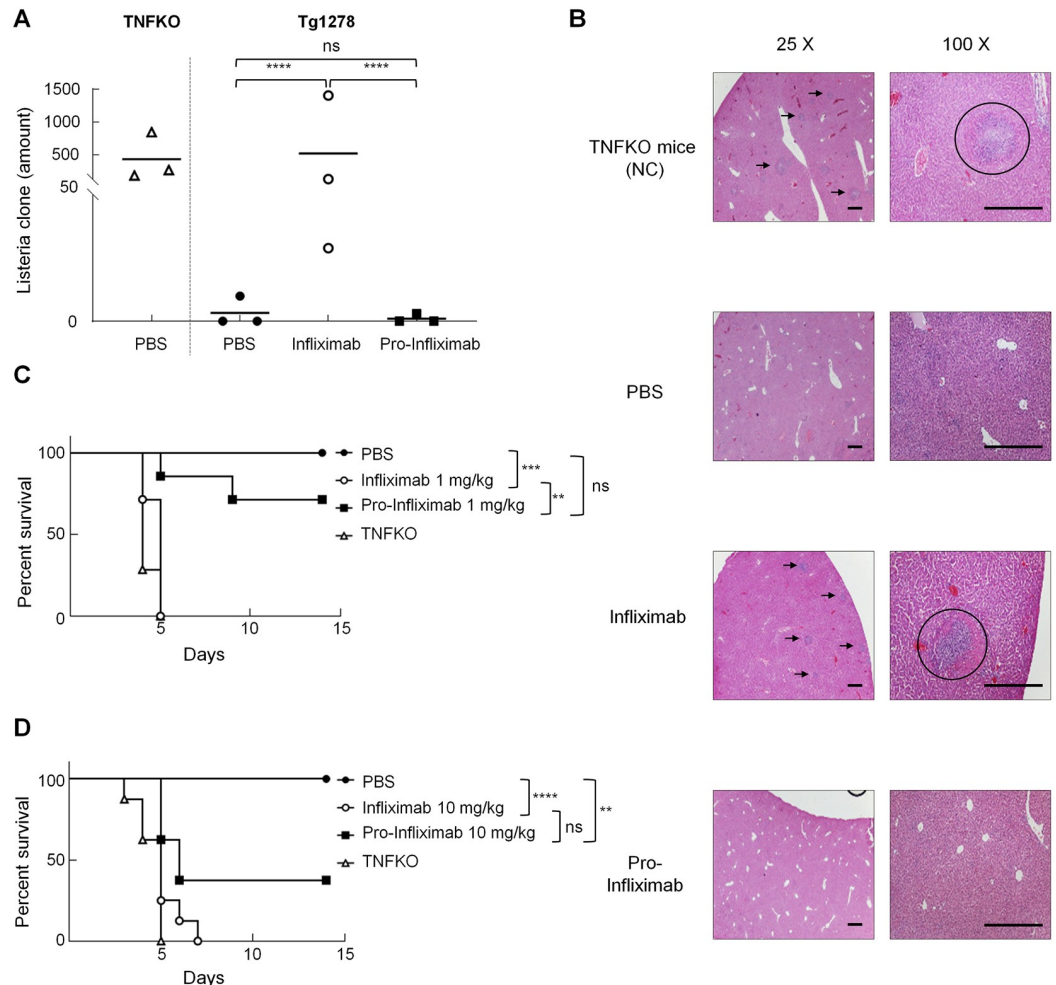


Fig 6. Comparison of the protective ability between pro-Infliximab and Infliximab on the host defense of TNFKO and Tg1278 TNFKO mice against *Listeria* infection. The Tg1278 mice ($n = 3$) were intraperitoneally injected with 1 and 10 mg/kg of pro-Infliximab, Infliximab, or control saline and challenged with lethal dosage of *L. monocytogenes* ($\times 10^4$ CFU) 2 h later. The blood CFU counts (A) and representative HE staining (B) of liver tissues were tested to compare the host defense of *Listeria* infection. The arrows and circles represent the sites of infection identified as big well-shaped granuloma structures containing necrotic hepatocytes and inflammatory cells. Bars = 500 μ m. (C and D) Survival rate after PBS, Infliximab, or pro-Infliximab treatment at low dose (1 mg/kg) (C) and high dose (10 mg/kg) (D) in *L. monocytogenes*-infected mice. The values are mean \pm SEM and the asterisks indicate a significant difference. ** $P < 0.01$, *** $P < 0.001$, **** $P < 0.0001$. Error bar: standard error of triplicate determinations. Underlying data can be found in [S1 Data](#). CFU, colony-forming unit; HE, hematoxylin–eosin; NS, no significance; Tg1278 mice, hTNF α -transgenic mice; TNF α , tumor necrosis factor α ; TNFKO, TNF α knockout.

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during circulation thoroughly, thus keeping its biological functions and preventing the undesirable blocking effect of TNF-dependent host defense mechanism, thereby maintaining part of host immunity during RA treatment.

Discussion

We have successfully developed an RA region, highly selective pro-Infliximab using an autologous hinge domain, which can significantly mask the TNF α -binding ability of Infliximab. This pro-Infliximab is selectively activated by disease-associated MMP-2/9 in the RA region and shows similar PKs and therapeutic efficacy as Infliximab without dysregulating systemic

immunity against *Listeria* infection in vivo. Additionally, the spatial hindrance-based Ab lock (Hinge) can prevent the neutralizing effect on antigen-binding ability of Infliximab from anti-Id Ab, which is a major problem in Ab-based therapy after repeating administration of Ab drugs. We believe that the MMP-cleavable and efficient Ab lock can significantly increase the selective reaction of Infliximab at the disease site and reduce the on-target toxicities of Infliximab during systemic circulation, thereby showing potential to improve the quality of life of RA patients.

Development of an Ab lock with high masking efficiency is important to enhance the selectivity of Ab drugs at the disease region long term. Unfortunately, no efficient strategy has hitherto been developed for improving selective activation of Infliximab at the RA region. Recently, Chen and colleagues attempted to solve this problem by creating a protease-activation pro-Infliximab with an inhibitory domain (latency-associated peptide [LAP]) that can be removed by proteases. [26] However, the LAP domain could only mask 1.86-fold of the TNF α -binding ability of the original Infliximab and significantly increased the molecular weight (MW) of pro-Infliximab (approximately 240 kDa) [26]. The poor masking efficiency and high MW of this pro-Infliximab may be insufficient to prevent the on-target toxicities and affect its PKs, respectively, during systemic administration of pro-Infliximab in RA patients. Additionally, the productive efficiency of this pro-Infliximab was significantly affected by the LAP domain and retained only 33% productive yield as compared with original Ab, [26] which led to difficulty in obtaining enough pro-Infliximab to investigate the PK, biodistribution, therapeutic efficacy, and on-target toxicities in an RA mouse model. In our study, we demonstrated that the Ab lock exhibits a 369-fold masking effect and only slightly increases the MW of Infliximab (approximately 160 kDa) (Fig 1A). The high masking efficiency and low MW means the Ab lock did not affect the productive yield and basic biological properties of pro-Infliximab and maintained the PK and biodistribution as control Infliximab in vivo (Fig 4). Our results also suggest that the pro-Infliximab has similar therapeutic efficacy and significantly decreases the risk of opportunistic infection in the RA mouse model as compared with Infliximab (Figs 5 and 6). Collectively, this is the first study to prove the concept that increasing the selectivity of the Ab drug at the disease site can reduce the risk of adverse effects whilst maintaining its therapeutic efficacy. We believe that the Ab lock is a novel and more suitable masking strategy for improving the selective activation of Infliximab at the RA region without altering its basic characteristics.

Blocking the binding of anti-Id Abs that influence the effectiveness of Ab drugs in RA treatment is very important because antidrug antibodies (ADAs, and mostly anti-Id Abs) have been reported to play a major role in causing a secondary response failure, in which the effectiveness of Ab drugs for RA therapy is lost over time despite a good initial response [27]. For example, 67% of patients with RA are treated with Adalimumab (fully human anti-TNF α Ab; Humira) developed ADAs within the first 28 weeks of treatment [28]. The antibodies against Infliximab can be detected in sera from 7% to 53% of Infliximab-treated RA patients [27]. The appearance of ADAs was associated with reduced serum concentrations of Infliximab and Adalimumab, decreased clinical response, and increased the risk of adverse events [19, 21]. Studies have also reported that 63% of patients with RA developed the anti-Campath (anti-CD52) Ab, with reduced therapeutic efficacy. [29] Here, we demonstrated that the spatial hindrance-based Ab lock can protect the complementarity-determining region (CDR) loop to prevent the response of the applied anti-Id Ab to Infliximab (anti-I-Id Ab); the binding of the anti-I-Id Ab to pro-Infliximab was 108-fold weaker than that to Infliximab (Fig 2). We also found that the binding ability of activated pro-Infliximab was not influenced by the application of the anti-I-Id Ab. These results suggest that with the Ab lock the pro-Infliximab has the potential to replace the Infliximab or serve as a second-line drug for RA patients with

anti-I-Id Ab. Concerns may be raised that the administration of pro-Ab could induce the production of anti-hinge Ab. We also analyzed the immunogenicity of pro-Infliximab by treating synthetic MMP-2/9 substrate sequence, Infliximab, or pro-Infliximab, respectively, to differentiated primary dendritic cells that coculture with CD4⁺ T cells (S5 Fig, the detailed experimental procedure can be found in S1 Text). The result indicated that there was no significant difference of CD4⁺ T cell proliferation between MMP-2/9 substrate sequence-treated, Infliximab-treated, pro-Infliximab-treated group, and control group. Moreover, IgG is the most abundant Ab in normal human serum, for about 10%–20% of plasma protein [30]. Furthermore, the IgG1 hinge domain is plentiful and derived from the Abs themselves. The autologous hinge should be the most appropriate Ab lock to form low immunogenicity pro-Ab drug.

TNF α -neutralizing Abs have been widely used to treat several autoimmune and immune-mediated disorders, such as RA [31], psoriasis [32, 33], and Crohn disease [34]. Among these diseases, proteases are attractive disease markers [35–37] and play important roles in various disease progression due to their high specificity and local activity [38]. The changeable design of protease substrates in the pro-Ab allows an Ab lock that can be widely applied to other diseases smoothly. For example, Infliximab is also used in the treatment of plaque psoriasis; we can replace the MMP-2/9 substrate linker with a marapsin substrate linker based on marapsin, which is a strongly up-regulated protease in psoriatic lesions [39]. In addition, there are several alternative Ab drugs for slowing the progression of RA by interrupting the immune process that promotes inflammation and joint damage. For instance, Tocilizumab (TCZ) is a humanized monoclonal Ab that acts against interleukin-6 receptor (IL-6R) for preventing the binding of proinflammatory cytokines (i.e., IL-6) to immune cells and is used for the treatment of moderate to severe RA disease [40]. Secukinumab (AIN457) was also developed for neutralizing IL-17A, which is a proinflammatory cytokine mainly secreted from T helper cells (Th-17) and currently in Phase III clinical trials for evaluation of its therapeutic efficacy and safety in RA treatment [41]. However, long-term inhibition of IL-6- or IL-17A-induced proinflammatory effects by Ab drugs may hamper the immunity of RA patients and increase the risk of adverse events such as serious tuberculosis infection [42] or fungal infection [43]. The spatial hindrance-based Ab lock is expected to be able to mask the antigen-binding site of a broad range of Ab drugs due to its highly conserved structure. Actually, we have also proved that the Ab lock could widely and significantly inhibit the antigen-binding ability of Ab drugs with different antigen specificities. For example, pro-anti-IL6 receptor Ab has an approximately 50.3-fold weaker antigen-binding ability than pro-anti-IL6 receptor Ab alone. (S6 Fig, the detailed experimental procedure can be found in S1 Text.) We believe that the universal Ab lock with changeable design of protease substrate can be applied to any alternative Ab drugs that are used for RA or even other disease treatment to improve their selective reactivity and clinical benefit.

In conclusion, we found that an autografted hinge can convert Infliximab into prodrug forms (pro-Infliximab) to improve the selectivity and reduce on-target toxicities of Infliximab. The pro-Infliximab has the following advantages: (1) it can be selectively activated in the RA region after cleaving by overexpressed protease; (2) it does not dysregulate the systemic immunity of TNF α ; (3) it does not change the productive yield, biodistribution, and PKs of the original Ab; (4) it may have the lowest immunogenicity because the hinge normally exists in human serum; and (5) it can prevent the neutralizing effects of anti-Infliximab idiotypic Ab and aid the realization of the full potential of Infliximab. We expect that this novel strategy for improving selective activation of Infliximab can significantly improve the quality of life in RA patients and cause revolutionary effect on the development industry of Ab drugs in the future.

Materials and methods

Ethics statement

DBA/1J, 1006 mice (mTNF α : +/+, hTNF α : +/-) were purchased from the Jackson Laboratory (Bar Harbor, ME, USA) and Taconic (Germantown, NY, USA), respectively.

Animal experiments were carried out in accordance with institutional guidelines and approved by the Animal Care and Use Committee of Kaohsiung Medical University, Kaohsiung, Taiwan (IACUC:104045). Tg197 mice (mTNF α : +/+, hTNF α : +/-), Tg1278 TNFKO (mTNF α : -/-, hTNF α : +/-), and TNFKO (mTNF α : -/-, hTNF α : -/-) mice were housed, maintained, and operated at Biomedcode (Vari, Greece) animal facilities, according to standard operating procedures. The Test Facility is accredited by the Hellenic Republic Prefecture of East Attica, Veterinary Service and Fishery Department and registered with the Ministry of Agriculture to conduct research in laboratory animals. All of the conditions of testing conformed to the Presidential Decree No. 160/1991 Governmental Gazette No. A' 64 applicable in Greece, which is the implementation of the EEC Directive 86/609/EEC, and were approved by the Veterinary Service Management of the Hellenic Republic Prefecture of East Attica (Approval license protocol No. 3720, 03/07/2013). The protocol was reviewed and approved by the Institutional Animal Ethical Committee at the Biomedical Sciences Research Centre "Al. Fleming" for compliance with regulations (Approval license No. 1125 on 23 February 2016). Due to the use of infectious material (*L. monocytogenes*), the study was performed in the Biosafety Level 2 facility of BSRC Al. Fleming, according to the relevant safety rules and regulations.

Pro-Ab construction, expression, and purification

The complementary DNA coding for the heavy and light chains of Infliximab were cloned through assembly PCR. Human IgG1 hinge sequences were obtained from the National Center for Biotechnology Information. The hinge-encoding sequences, GGGGS linker, and MMP-2/9 substrate-encoding sequences (GPLGVV) [44] were introduced upstream of the light chain and heavy chain of Infliximab to generate pro-Infliximab. Infliximab or pro-Infliximab production were through the Expi293 Expression System (Thermo Fisher Scientific, Waltham, MA, USA) and purified by Protein A-Sepharose (GE Healthcare, Milwaukee, WI, USA).

Comparison of the binding ability of pro-Infliximab with or without MMP-2/9 treatment

To determine the binding kinetics (EC_{50}) of pro-Infliximab and Infliximab, TNF α was coated onto 96-well plates and blocked with 5% skim milk. Infliximab or pro-Infliximab was incubated with or without 20 μ g/mL of MMP-2/9 (type IV collagenase, Sigma-Aldrich, St. Louis, MO, USA) in DMEM/0.05% BSA (pH 7.4) for 1 h at 37 °C before the reaction was terminated by BCS. All of the samples were added onto the plates at the given concentrations (500–0.001 nM) for 1 h at RT. After washing, the wells were incubated with HRP-goat anti-human IgG Fc γ Ab for 1 h at RT, and detection was performed by the addition of ABTS containing 30% H₂O₂ (Sigma-Aldrich). The binding ability was quantified through absorbance detection at 405 nm.

Neutralization of TNF α signal by pro-Infliximab

Human embryonic kidney cell line (HEK293) cells were transiently transfected with the luciferase reporter plasmid pNF- κ B-Luc [45] (BD Biosciences Clontech, Palo Alto, CA, USA), internal control Renilla-Luc reporter plasmid, or positive control pFC-MEKK plasmid with

TransIT-LT1 reagent (Mirus Bio, Madison, WI) for 6 h. After the transfection procedure, the medium was replaced with TNF α (20 ng/mL, Sigma-Aldrich) and incubated with Infliximab, pro-Infliximab, MMP-2/9 preincubated Infliximab, or MMP-2/9 preincubated pro-Infliximab for 1 h at 37 °C. After removing from the medium, the cells were cultured for 16 h at 37 °C, and cell extracts from each sample were measured using the dual-luciferase reporter assay system (Promega, Madison, WI, USA) according to the manufacturer's protocol.

Evaluation of the binding ability of anti-Id Ab to pro-Infliximab

Anti-Id Ab (0.3 μ g/mL, Bio-Rad Laboratories, Redmond, WA, USA) was coated onto 96-well plates and blocked with 5% skim milk. Infliximab-biotin or pro-Infliximab-biotin was incubated with or without 25 mg of MMP-2/9 in DMEM/0.05% BSA (pH 7.4) for 1 h at 37 °C before the reaction was terminated by BCS. All the samples were added onto the plates at the given concentrations for 1 h at RT. After washing, the plate was sequentially incubated with HRP-conjugated streptavidin (Jackson ImmunoResearch Laboratories). The detection was performed by the addition of ABTS containing 30% H₂O₂. The binding ability was quantified through absorbance detection at 405 nm.

To investigate the effect of anti-Id Ab on the restoration ability of TNF α binding of pro-Infliximab by protease, 96-well plates were coated with Infliximab and pro-Infliximab for 2 h at 37 °C and blocked with 5% skim milk. The plates were incubated with different concentrations (8–1,000 ng/mL) of anti-Id Ab for 1 h, then unbound anti-Id Ab was removed with PBST. Each sample was incubated with or without 20 μ g/mL of MMP-2/9 for 1 h. After washing, each sample was incubated with TNF α -biotin for 1 h. The plates were washed with PBS and sequentially incubated with HRP-conjugated streptavidin. The procedures were performed as described previously.

Measurement of the serum half-life and biodistribution in vivo

DBA/1J mice ($n = 5$) were injected intraperitoneally with 50 μ Ci ¹³¹I-labeled pro-Infliximab or 50 μ Ci ¹³¹I-labeled Infliximab. Whole blood was collected via the tail vein at different time points. The organ specimens were harvested at 12, 24, 96, and 168 h points postadministration of radio-labeled antibodies. The blood and tissue were weighed on an analytical balance and assayed for radioactivity in a multichannel gamma counter. The initial and terminal half-life of the probes were estimated by fitting the data to a two-phase exponential decay model with Prism software (Graphpad Software, San Diego, CA).

Removal of the inhibitory domain from pro-Infliximab through MMP-2/9 treatment or MMP-2/9 in the disease region

Human TNF α -transgenic 1006 mice [46] (mTNF α : +/+, hTNF α : +/-) can develop spontaneous arthritis at about 7 or 8 weeks of age. Fourteen-week-old 1006 mice with moderate disease were intraperitoneally injected with 50 μ g/mouse Infliximab or pro-Infliximab. After 24, 48, 96, and 168 h, the mice were sacrificed and blood, colon, lung, spleen, and hind paw specimens were collected. All tissues were homogenized and centrifuged to collect the supernatant and the light-chain MW profile of pro-Infliximab and Infliximab in the hind paw, and serum samples were then evaluated by western blot with HRP- α hIgG Fab Ab (Jackson ImmunoResearch Laboratories, West Grove, PA, USA). β -actin was detected by rabbit anti β -actin Ab (Cell Signaling Technology; Boston, Massachusetts, USA) and HRP-conjugated anti-rabbit IgG (H+L) Ab (Invitrogen, Carlsbad, CA, USA).

Therapeutic efficacy of pro-Infliximab for rheumatoid arthritis in vivo

The 6-week-old Tg197 mice [47] ($n = 8$) were intraperitoneally injected with 10 mg/kg Infliximab, 10 mg/kg pro-Infliximab, or saline. All mice were dosed twice weekly for 6 weeks. The therapeutic efficacy of each group was monitored every week and evaluated using weight measurements and clinical scores. Clinical scores were based on a previously described scoring system [48]. After treatment for 6 weeks, all mice were sacrificed, and the hind ankle joints were removed for histology. The mouse paws were removed and fixed with formalin buffer for HE staining, and the histopathologic score was evaluated microscopically, as described previously [22], using a modified scoring system.

Comparison of on-target toxicities of pro-Infliximab and Infliximab in vivo

The Tg1278 mice, which can only express normally regulated hTNF α but lack mTNF α [25], were intraperitoneally injected with 1 and 10 mg/kg of pro-Infliximab, Infliximab, or control saline and challenged with lethal dosage of *L. monocytogenes* ($\times 10^4$ CFU) 2 h later. After 48 h, the three mice per group were sacrificed, and blood samples were collected for *Listeria* CFU determination while liver tissues were harvested for histopathological assessment; the other mice ($n = 7$) were monitored daily, and their survival was recorded for a total period of 2 weeks.

Statistical analysis

Data are presented as mean \pm SEM or SD. The protective effect of pro-Infliximab and Infliximab on pathological progression of RA and the mean body weight of Tg197 mice were analyzed with two-tailed Mann–Whitney test or unpaired *t* test, respectively, and the number of *Listeria* CFUs in the blood specimens was analyzed with F-test to compare the statistical significance of the differences between the controls and samples. Statistical analysis was performed using the GraphPad Prism v.6, and data were considered significant at a *P* value of less than 0.05.

Supporting information

S1 Fig. MW of pro-Infliximab and Infliximab with MMP-2/9 for different lengths of time.

To investigate whether the Ab lock can be efficiently removed from pro-Infliximab and restore the antigen-binding ability of pro-Infliximab after MMP-2/9 cleavage, we incubated pro-Infliximab and Infliximab with MMP-2/9 for different time periods and evaluated the MW profile of Ab fragments by western blot. The results showed that the heavy-chain (58 kDa) and light-chain (29.4 kDa) molecular weight of pro-Infliximab was converted into a profile similar to the control Infliximab (55 kDa for heavy chain and 25.6 kDa for light chain) after treatment with MMP-2/9 within 60 min, demonstrating that MMP-2/9 could completely remove the Ab lock from pro-Infliximab. Ab, antibody; MMP, matrix metalloproteinase; MW, molecular weight.

(TIF)

S2 Fig. TNF α binding of pro-Infliximab and Infliximab with MMP-2/9 for different lengths of time.

The TNF α -binding ability of pro-Infliximab was gradually elevated in a time-dependent manner during MMP-2/9 treatment and finally restored to a level similar to the control Infliximab. MMP, matrix metalloproteinase; TNF α , tumor necrosis factor α .

(TIF)

S3 Fig. The activation of pro-Infliximab in peripheral organ. hTNF α -transgenic 1006 mice were intraperitoneally injected with 50 μ g Infliximab or pro-Infliximab. After 24, 48, 96, and 168 h, the (A) lung, (B) colon, and (C) spleen tissue were collected using HRP-conjugated anti-human IgG Fc Ab for detecting the level of active and inactive pro-Infliximab by western blot. The β -actin as internal control. Ab, antibody; Fc, fragment crystallizable; HRP, horseradish peroxidase; IgG, immunoglobulin; TNF α , tumor necrosis factor α . (TIF)

S4 Fig. Effect of pro-Infliximab and Infliximab on the mean body weight of Tg197 mice. By the end of the study (11 weeks of age), the mean body weights of all groups treated twice weekly from week 6 were as follows: PBS = 18.10 ± 1.54 g, Infliximab 10 mg/kg = 24.41 ± 1.37 g, and pro-Infliximab 10 mg/kg = 22.57 ± 1.64 g. Error bars indicate standard error of the mean. Tg197 mice, hTNF α -transgenic mice; TNF α , tumor necrosis factor α . (TIF)

S5 Fig. Immunogenicity of human immune cells to Infliximab, pro-Infliximab, and MMP-2/9 SL. We cocultured dendritic cells differentiated from human PBMCs with autologous CD4⁺ T cells and stimulated with control medium (represented as DC+T), PHA (as positive control), Infliximab, pro-Infliximab, or MMP-2/9 SL, respectively, for 5 days. Then, we detected the proliferation of CD4⁺ T cells by ATPlite Luminescence Assay kit (Perkin Elmer). Bars, SD. CPM, counts per minute; MMP, matrix metalloproteinase; PBMC, peripheral blood mononuclear cell; PHA, phytohemagglutinin; SL, substrate linker. (TIF)

S6 Fig. The Ab lock inhibits the IL-6R-binding ability of pro-anti-IL6 receptor Ab. The IL-6R-binding ability were analyzed by antigen based ELISA. The EC₅₀ of anti-IL6 receptor Ab, pro-anti-IL6 receptor Ab, and MMP-2/9-activated pro-anti-IL6 receptor Ab were 1.77 nM, 88.97 nM, and 2.827 nM. Ab, antibody; EC₅₀, half-maximal effective concentration; IL-6R, interleukin-6 receptor; MMP, matrix metalloproteinase. (TIF)

S1 Text. This file contains supplemental methods and references. (DOCX)

S1 Data. This file contains the raw data presented in figures in the main manuscript (Figs 2–6) and supplemental figures (S2, S4, S5 and S6 Figs). (XLSX)

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