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# Transport patterns of anti-TNF-α in burn wounds: Therapeutic implications of hyaluronic acid conjugation

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ABSTRACT

A central complication in burn injuries is progression of the zone of necrosis, which is associated with intense inflammatory responses. Conjugation of monoclonal antibodies against tumor necrosis factor-α (TNF-α), a central mediator of inflammation, to high molecular weight hyaluronic acid (HA) has been shown to be an effective treatment in reducing secondary necrosis in rodent models of deep partial-thickness burns. Here the transport of conjugated and non-conjugated antibodies in burn injuries was investigated to explore the effects of antibody tethering on the spatiotemporal distribution of anti-TNF-α. Diffusion constants were measured in solution and in type I collagen gels *in vitro* using fluorescence correlation spectroscopy to provide quantitative comparisons of the effects of conjugation. It is shown that the HA significantly increased the antibody residence time in the superficial region at 24 h in burn injuries, which strongly correlated with the pattern of inflammatory cell infiltrate in the tissue. A transport model was used to fit the results of antibody distribution in the tissue based on fluorescence correlation spectroscopy measurements, resulting in estimates for effective diffusion constants that demonstrate the effects of HA conjugation on the biodistribution of therapeutic proteins. These results demonstrate that tuning residence time of therapeutic proteins can be an effective strategy in regulating the inflammatory response associated with acute injuries.



## 1. Introduction

Numerous biomaterial strategies have been developed for the targeted delivery of biologics to treat injuries and inflammatory diseases, including degradable microspheres, injectable scaffolds and microneedle arrays [1–4]. Conjugation of therapeutic antibodies to high molecular weight hyaluronic acid (HA) has been shown to be an effective strategy for administering inhibitors of the pro-inflammatory cytokine tumor necrosis factor-α (anti-TNF-α) [5], but there is a lack of mechanistic understanding when it has been shown that direct application of non-conjugated antibodies is only effective at reducing inflammatory responses in wounds at doses that are 100-times higher [6].

Burn injuries represent an exceedingly complex inflammatory cascade in which an intense, local inflammatory response can result in injury progression, characterized by the formation of secondary necrosis, while the systemic immune response is often characterized by immunosuppression and pronounced susceptibility to infections [7]. This microenvironment is thought to make regenerative therapies based on bioactive scaffolds or stem cells inherently less effective due to the high concentrations of matrix-degrading enzymes and inflammatory mediators that can drive cellular necrosis [8]. However, despite the complexity of these injuries, they are a prime candidate for the types of localized therapeutic treatments developed for delivery of anti-inflammatory drugs, which could treat the local symptoms but not antagonize the systemic ones.

Previous studies on treatment of deep partial-thickness burns in rats with HA conjugated anti-TNF-α ((anti-TNF-α)-HA) have shown a 70% reduction in secondary necrosis which inversely correlated with increased re-epithelialization distances (Fig. 1a). Localization of anti-TNF-α via conjugation to HA also led to differences in the spatial distribution of inflammatory cells attracted by positive concentration gradients of TNF-α such as macrophages (Fig. 1c) and polymorphonuclear cells (Fig. 1d) [9–11]. The effects were assumed

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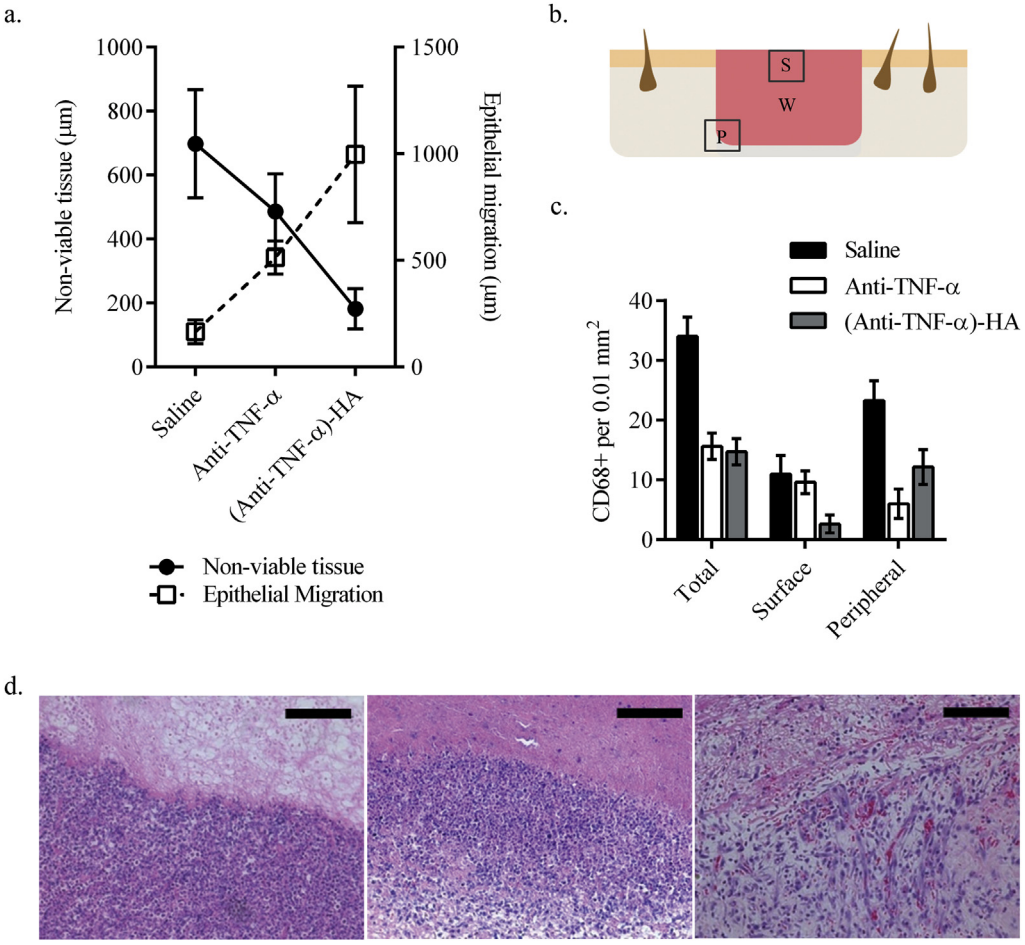


**Fig. 1.** Motivation of present study. (a) Previous work showed efficacy of topical application of (anti-TNF-α)-HA in the reduction of secondary necrosis and in the increase in re-epithelialization at day 7. (b) Spatial dependence of cell infiltration in the wound was observed in images taken at either the surface (S) or periphery (P) of the wound bed (W). Cell numbers per area were tallied for $CD68^{+}$ cells at day 4 in saline control, anti-TNF-α and (anti-TNF-α)-HA treated wounds (c) to illustrate the differences in antibody transport affecting macrophage counts in the deep periphery, surface of the wound bed or sum of both areas. (d) Similar differences in density of polymorphonuclear cells at the surface of the wound bed is seen in saline control (left), anti-TNF-α (middle), and (anti-TNF-α)-HA (right) treated wounds. Error bars are mean ± SD; Scale bars are 100 μm.

to rely on the increased residence time of anti-TNF-α at the injury site due to HA conjugation, which was further assumed to be localized on the surface of the burn injury. The conjugates likely diffuse through the burn site at a rate which is dependent on the HA molecular weight, which decreases as a function of time due to enzymatic and oxidative degradation and ultimately results in longer residence times and a broad distribution throughout the granulation tissue. Such transport dynamics would create complex, time-dependent distributions of anti-TNF-α that could have significant relevance to the ultimate therapeutic efficacy of these conjugates.

Based on our previous burn work, a basic model of TNF-α transport processes in burned tissue treated with (anti-TNF-α)-HA was proposed, which assumes that the (anti-TNF-α)-HA remains on the top of the wound, acting as a sink for TNF-α [10]. While this model was consistent with basic effects, such as antibody dose requirements, it may not entirely represent what is observed in *in vivo* experiments for the 2 day duration of a treatment between doses. Experimentally, there appear to be two distinct phases, one in which the conjugate resides on top of the wound and acts as a sink for TNF-α, and another as the conjugate degrades and slowly diffuses into the wound bed. We therefore sought to determine the diffusion rates and transport properties of (anti-TNF-α)-HA into the wound bed.

Here we measure the spatial distribution of (anti-TNF-α)-HA conjugates in a rat burn model and compare this with non-conjugated antibody using quantitative fluorescence microscopy, demonstrating that while non-conjugated antibody diffuses through the superficial region of granulation tissue within several hours, (anti-TNF-α)-HA persists on the order of days. These results were consistent with diffusion measurements in collagen matrices, which showed significant reductions in antibody mobility due to HA conjugation. This altered antibody distribution pattern is correlated with changes in inflammatory-cell infiltrate, suggesting the increased residence time of this therapeutic is critical for rescuing burn tissue from inflammation-induced necrosis.

## 2. Methods

### 2.1. Material preparation and characterization

#### 2.1.1. Conjugation and fluorescent labeling

The conjugation reaction followed procedures previously described by our lab [9–11]. Conjugation of anti-TNF-α to HA was achieved through dehydrative coupling of free carboxylic acid groups on HA to free amines on the antibody with a ratio of HA chains:antibody of 21:1. High molecular weight hyaluronic acid ($M_w$ = 1.6 MDa, Sigma Aldrich, St. Louis, MS) was dissolved overnight at room temperature in Millipore water to a final concentration of 10 mg/mL. A 500 μg vial of lyophilized antibody was