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ARTICLE

Codelivery of antigen and an immune cell adhesion inhibitor is necessary for efficacy of soluble antigen arrays in experimental autoimmune encephalomyelitis

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Autoimmune diseases such as multiple sclerosis (MS) are typified by the misrecognition of self-antigen and the clonal expansion of autoreactive T cells. Antigen-specific immunotherapies (antigen-SITs) have long been explored as a means to desensitize patients to offending self-antigen(s) with the potential to retolerize the immune response. Soluble antigen arrays (SAgAs) are composed of hyaluronic acid (HA) cografted with disease-specific autoantigen (proteolipid protein peptide) and an ICAM-1 inhibitor peptide (LABL). SAgAs were designed as an antigen-SIT that codeliver peptides to suppress experimental autoimmune encephalomyelitis (EAE), a murine model of MS. Codelivery of antigen and cell adhesion inhibitor (LABL) conjugated to HA was essential for SAgA treatment of EAE. Individual SAgA components or mixtures thereof reduced proinflammatory cytokines in cultured splenocytes from EAE mice; however, these treatments showed minimal to no *in vivo* therapeutic effect in EAE mice. Thus, carriers that codeliver antigen and a secondary "context" signal (*e.g.*, LABL) *in vivo* may be an important design criteria to consider when designing antigen-SIT for autoimmune therapy.

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INTRODUCTION

Advancements in treating autoimmune diseases such as multiple sclerosis (MS) have been made by enhancing our understanding of the molecular processes involved in disease progression.[1,2] The complexity of immune responses and the heterogeneous nature of immune cells complicate the design of therapeutics. In the case of relapsing-remitting MS, the disease state is propagated through professional antigen-presenting cells, stimulating the clonal expansion and activation of T cells recognizing endogenous autoantigen(s) resulting in subsequent attack(s) on the central nervous system and neural degeneration.[3–5] The majority of current US Food and Administration (FDA)–approved therapies for MS focus on suppressing disease symptoms through inhibition of the immune inflammatory response.[6–8] In fact, most therapeutics may manage disease symptoms, but are broadly immunosuppressive, often leading to substantial side effects.[9]

"Antigen-specific immunotherapies" (antigen-SITs) aim to reprogram the immune response, which is the root cause of many autoimmune diseases.[10] An FDA-approved MS therapy, Copaxone (R) (Teva Neuroscience, Kansas City, MO), utilizes an antigen-specific approach using polymeric antigen derived from myelin basic protein in an effort to promote tolerance by inducing antigen-specific regulatory T cells.[11–13] Although the mechanisms of Copaxone (R) are still under active investigation, it has been shown to improve clinical outcomes in patients and in animal models of MS such as experimental autoimmune encephalomyelitis (EAE).[14]

Further therapeutic enhancement of antigen-SITs may be achieved by codelivering another active molecular signal (*i.e.*, adjuvant). Vaccines provide an interesting parallel to the concept of adjuvanted antigen-SITs. In traditional vaccines, antigen presentation and recognition result in immune protection through use of adjuvanted formulations.[15–18] Protection is achieved by presenting antigen with a second proinflammatory "context" signal to direct appropriate protective immune responses. Administration of antigens without adjuvant can also induce immune tolerance. For example, frequent low doses of soluble antigen have been used to induce tolerance in patients to treat specific allergies.[19–22] Both vaccine and antigen-SIT approaches are "antigen specific;" however, the dose and the presence or absence of a "secondary" signal (*e.g.*, vaccine adjuvant) drastically alter the response from proinflammatory to tolerogenic. Simultaneous presentation of antigen and another "secondary" (coinhibitory) signal using peptide conjugates has also been shown to skew the immune response toward tolerance.[23–30] Thus, both antigen and codelivery of a secondary signal (*i.e.*, adjuvant, immune cell inhibitor) are essential for determining the resultant immune response.[31,32]

History also provides some guidance for designing a molecular construct to codeliver antigen and a secondary signal. Beginning in 1976, Dintzis and others systematically studied a wide range of

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polymers with grafted "haptens" (*e.g.*, antigens) and developed a set of rules for inducing immune stimulation or tolerance.[33,34] "Dintzis Rules" proposed that characteristics such as polymer size, solubility, flexibility, antigen valency, spacing, and binding avidity are necessary to direct the immune response. Of particular interest, carriers under 100 kDa could be tolerogenic and, depending on hapten density, could target or induce a subset of immune cells (T cells or B cells) directly.[35–37] Such thinking prompted rational design of graft polymers that simultaneously display multiple copies of antigen and a secondary "context" signal that could inhibit costimulation.

Here, Dintzis Rules (polymers < 100 kDa displaying ~1–2 antigens per 1,000 kDa) were combined with the concept of utilizing this carrier to codeliver antigen and an immune cell adhesion inhibitor, which was previously shown to suppress EAE, a murine model of MS.[38] Soluble antigen arrays (SAgAs) were synthesized by cografting a known MS autoantigen derived from proteolipid protein amino acids 139–151 (proteolipid protein peptide (PLP)) and an intercellular adhesion molecule 1 (ICAM-1) inhibitor peptide (LABL, ITDGEATDSG) to hyaluronic acid (HA) via a single-step grafting technique (Table 1).[38] Once efficacy was demonstrated, SAgA containing both grafted peptides ($SAgA_{PLP:LABL}$) was compared to a variety

Table 1 Sample identification, peptide concentration, and number of peptides per HA (16.9 kDa) chain as determined by HPLC

Sample	*PLP conc. (nmol)*	*LABL conc. (nmol)*	*Final ratio*	*Number of peptides per HA polymer*
$SAgA_{PLP:LABL}$	275	325	1.2:1	11:10 (LABL:PLP)
HA_{LABL}	—	462	N/A	15
HA_{PLP}	286	—	N/A	11

The peptide concentrations were calculated based on HPLC analysis of 1-mg complex. Results shown are an average from triplicate injections of a single batch preparation. All samples had a relative standard deviation (RSD) ≤0.01.
Conc., concentration; HA, hyaluronic acid; HA_{LABL}, HA with grafted LABL peptide; HA_{PLP}, HA with grafted PLP peptide; HPLC, high-performance liquid chromatography; N/A, not applicable; PLP, proteolipid protein peptide; SAgA, soluble antigen array; $SAgA_{PLP:LABL}$, SAgA cografted with PLP and LABL peptides.

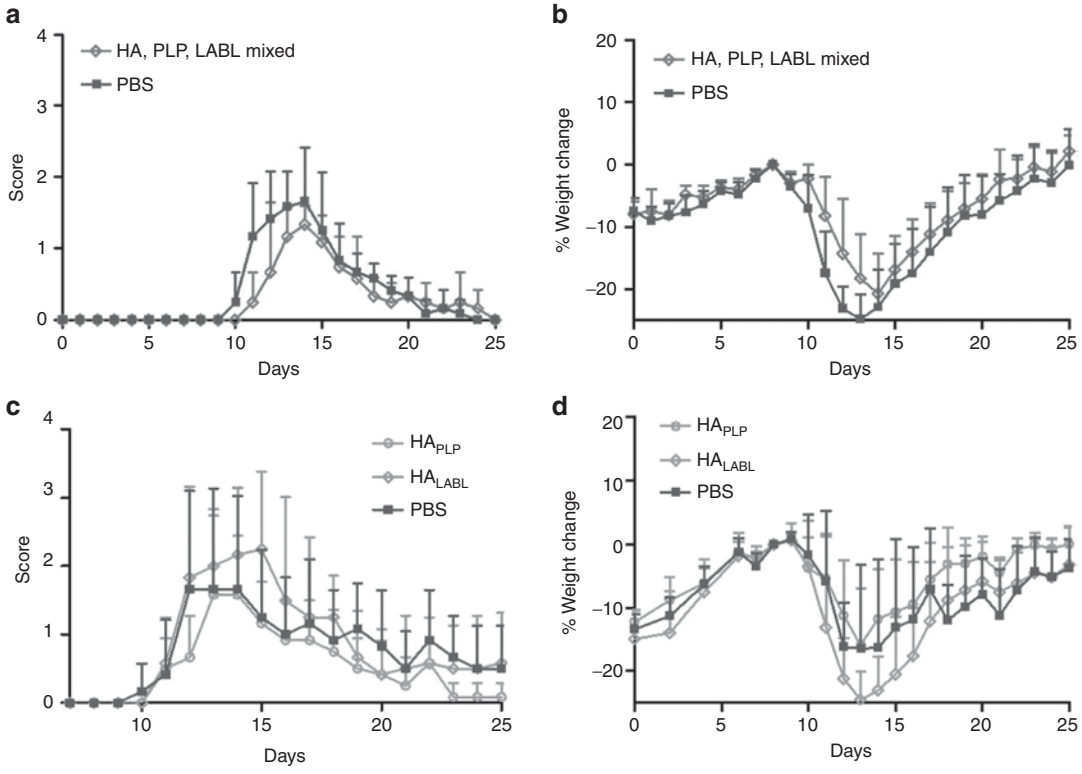

Figure 1 Experimental autoimmune encephalomyelitis (EAE) is not affected by component mixture or hyaluronic acid (HA)-conjugate controls. EAE was induced in SJL mice (day 0) and were subsequently treated with (**a**,**b**) component mixture of HA, free proteolipid protein peptide (PLP), and free LABL peptides and (**c**,**d**) HA with grafted PLP peptide (HA_{PLP}) and HA with grafted LABL peptide (HA_{LABL}) polymer controls on days 4, 7, and 10. All samples showed no statistical difference when compared with phosphate-buffered saline (PBS) control group. Data are expressed as mean ± SD, $n = 6$ mice per group.

of therapeutic controls to verify that disease suppression was due to simultaneous multivalent presentation of both antigen and cell adhesion signals. The effect of $SAgA_{PLP:LABL}$ on splenocytes isolated from EAE mice was also investigated and compared with *in vivo* treatment of EAE mice.

RESULTS

Characterization of polymeric SAgAs and HA graft control molecules

Peptides were manufactured using solid phase synthesis, and the HA polymer backbone was purchased from Lifecore. Gel permeation chromatography and high-performance liquid chromatography (HPLC) were used to characterize the manufactured peptides, the homopeptide graft polymer controls (HA with grafted LABL peptide (HA_{LABL}) and HA with grafted PLP peptide (HA_{PLP})), and the $SAgA_{PLP:LABL}$ as previously reported.[38] The expected shift in retention time was observed by gel permeation chromatography, suggesting an increase in molecular weight relative to the HA starting material, as compared with pullulan standards (see Supplementary Figure S1). A graphical representation of each of the prepared samples and the calculated amount of peptide grafted to the polymer determined by HPLC are shown in Table 1. HPLC data showed a consistent concentration of grafted peptides for all samples. Peptide concentration was calculated based on analysis of 1-mg manufactured complex and comparison with a standard curve for the individual peptides. $SAgA_{PLP:LABL}$ samples had similar peptide concentration as HA_{PLP} and HA_{LABL} controls, but the homopolymers displayed half the amount of total peptide as indicated by values in Table 1.

Codelivery of conjugated antigen and inhibitor represses EAE disease *in vivo*

A control component mixture (HA, free LABL, and free PLP), graft homopolymer controls (HA_{LABL}, HA_{PLP}, and a 1:1 mixture of HA_{LABL} and HA_{PLP}), and $SAgA_{PLP:LABL}$ were evaluated in the PLP:SJL/J mouse EAE model against a phosphate-buffered saline (PBS) negative control. All samples were dosed so that the concentration of PLP delivered was equal (200 nmol). A dose-ranging study supported the use of 200 nmol of PLP (see Supplementary Figure S2). For HA_{LABL} samples, LABL peptide content was calculated to equal the LABL content in $SAgA_{PLP:LABL}$ (*i.e.*, 200 nmol of LABL). Disease onset occurs ~10–12 days postimmunization. At this time, mice begin to show disease signs such as weakness, paralysis of their tail and limbs, and loss of body weight.

Disease was not altered in mice treated with the component mixture, HA_{LABL}, or HA_{PLP} homopolymer controls as indicated by clinical score and percent weight change (Figure 1a–d). In contrast, mice treated with a 1:1 mixture of HA_{LABL} and HA_{PLP} homopolymers or the cografted $SAgA_{PLP:LABL}$ showed significant disease suppression (Figure 2). The $SAgA_{PLP:LABL}$ treatment significantly suppressed EAE ($P < 0.05$) in both score and percent weight change on days 11–17 of the study, whereas the 1:1 homopolymer mixture of HA_{LABL} and HA_{PLP} significantly inhibited disease ($P < 0.05$) on days 11–15. While the $SAgA_{PLP:LABL}$ treatment showed longer disease inhibition compared with PBS, this treatment was not significantly different from the mixture of HA_{PLP} and HA_{LABL}.

$SAgA_{PLP:LABL}$ treatment alters cellular metabolism and cytokine expression during peak of disease in an *ex vivo* PLP rechallenge model

In order to identify whether the *in vivo* protection afforded by $SAgA_{PLP:LABL}$ altered splenocyte population characteristics, cellular metabolism of harvested splenocytes was measured. To achieve this, mice were induced with EAE (clinical scoring data not shown), and splenocytes were isolated on day 12 (peak of disease) and day 25 (disease remission). *Ex vivo* stimulation studies of EAE splenocytes isolated on day 12 or 25 suggested that the day 25 EAE splenocytes had a more robust response to PLP rechallenge. This is demonstrated by the statistical increase in metabolic and cytokine values shown in Table 2. Therefore, the day 25 time point was used to identify whether $SAgA_{PLP:LABL}$ or control treatments could differentially alter cellular metabolism and cytokine expression in PLP rechallenged EAE splenocytes.

The harvested splenocytes were then treated *ex vivo* and rechallenged with 25 µmol/l PLP antigen or vehicle. Basal levels of cellular metabolism were not altered between $SAgA_{PLP:LABL}$- and PBS-treated mice (Figure 3). Additionally, cellular metabolism was not further altered by the PLP rechallenge in all samples except HA_{LABL}, where PLP rechallenge resulted in a large increase in resazurin fluorescence reading (Figure 3).

In vitro cytokine profiling in $SAgA_{PLP:LABL}$ and conjugate control treated splenocytes

Whether the difference in cellular metabolism was coupled to cytokine response in these splenocyte populations was explored next. *In vitro* cytokine levels were determined 120 hours posttreatment

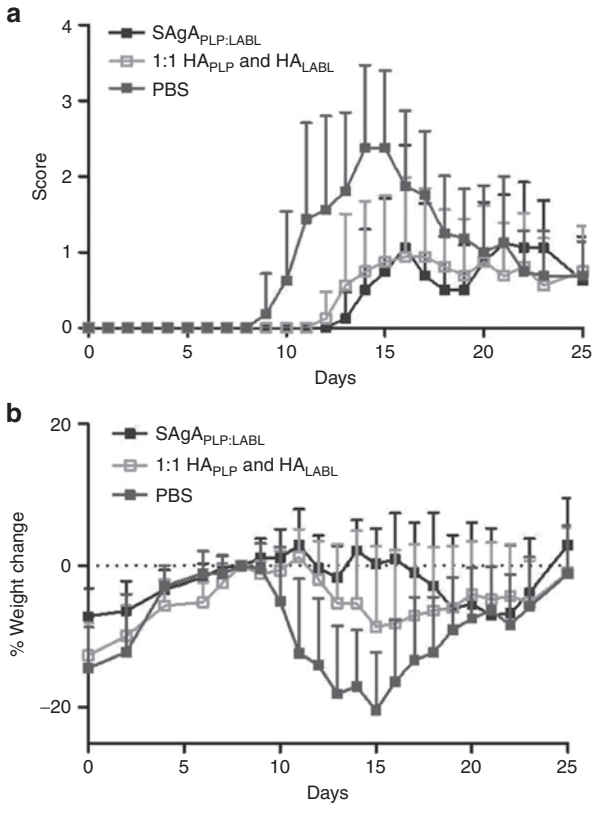

Figure 2 Codelivery of conjugated proteolipid protein peptide (PLP) antigen and conjugated LABL peptide improves experimental autoimmune encephalomyelitis (EAE) clinical outcomes. EAE was induced in SJL mice (day 0) and were subsequently treated with soluble antigen array cografted with PLP and LABL peptides ($SAgA_{PLP:LABL}$) or a 1:1 mixture of hyaluronic acid (HA) with grafted PLP peptide (HA_{PLP}) and HA with grafted LABL peptide (HA_{LABL}) polymer controls on days 4, 7, and 10. Daily (**a**) clinical scores ($P < 0.05$; days 11–17) and (**b**) percent weight change was determined ($P < 0.05$; days 11–17). Data are expressed as mean ± SD, $n = 6$ mice per group. PBS, phosphate-buffered saline.

by using bead array enzyme-linked immunosorbent assays (ELISAs). A total of nine cytokines showed significant differences between media treatment and $SAgA_{PLP:LABL}$ and control treatments. Treatment with HA_{LABL} significantly reduced basal and PLP-induced cytokine levels of all cytokines profiled, except for interleukin (IL)-2, which seemed to be not altered. For all other treatments, only $SAgA_{PLP:LABL}$ showed a significantly lower level of IL-4 as compared with the media, and no difference was seen in the level of IL-5. The data for IL-6 showed that while basal levels of IL-6 were elevated with sample treatment over media controls, levels after PLP rechallenge remained constant and were significantly decreased than that of rechallenged media control samples. Conversely, treatment with HA_{LABL}, the mixture of free HA and free peptides, and the mixture of HA_{LABL} and HA_{PLP} significantly increased the levels of IL-2 in PLP restimulated samples as compared with the respective basal media control. The 1:1 mixture of HA_{PLP} and HA_{LABL} showed significant increases in both the basal and restimulated samples.

Cytokines associated with allergic and autoimmune inflammatory responses, IL-13, IL-17, IL-22, and tumor necrosis factor-α (TNF-α), were also measured (Figure 4). Similar to our previous study, data for interferon-γ (IFN-γ) in PLP rechallenged splenocytes at day 25 were above the maximum level of detection, and conclusions could not be drawn from the data set (see Supplementary Figure S2). In this set of cytokines, HA_{LABL} again showed significantly decreased levels compared with the media control even when rechallenged with PLP. For all other samples, a significant increase in basal levels of IL-13 and TNF-α was seen. Upon rechallenge with PLP, a significant decrease in IL-13 expression was seen when treated with the HA_{LABL} and HA_{PLP} mixture and $SAgA_{PLP:LABL}$. Similarly, increased levels of IL-22 were seen in all samples as compared with media basal levels; however, only the HA_{LABL} and HA_{PLP} mixture provided a significantly decreased level of IL-22. The data suggested that HA_{LABL} represses and in most cases eliminates cytokine expression of most cytokines profiled. Furthermore, the data suggested that variations of peptide carriers can alter cytokine response in cultured EAE splenocytes.

DISCUSSION

It was rationalized that combining delivery of antigen with delivery of a secondary "context" signal, in this case a peptide known to inhibit immune cell adhesion, may synergize the effects of two discrete therapeutic approaches: presentation of antigen and interruption of cell signaling events. Much like vaccines, which codeliver antigen and immune-stimulatory adjuvant to illicit and immune response, it was theorized that codelivery of antigen and an inhibitor of immune cell adhesion should reintroduce tolerance to the presented antigen, opening new opportunities to antigen-SITs. In this study, we evaluated SAgAs as novel antigen-SIT molecules that are covalently grafted with cell adhesion antagonists and the causal

Table 2 Cellular metabolism and culture supernatant cytokine expression levels in EAE splenocytes were significantly higher at disease remission (day 25) than at peak of disease (day 12)

Isolation day	*12*		*12*		*25*		*25*	
$SAgA_{PLP:LABL}$ ex vivo PLP stimulation	*PBS*		*$SAgA_{PLP:LABL}$*		*PBS*		*$SAgA_{PLP:LABL}$*	
	Vehicle	*25 µmol/l PLP*	*Vehicle*	*25 µmol/l PLP*	*Vehicle*	*25 µmol/l PLP*	*Vehicle*	*25 µmol/l PLP*
Resazurin (ex560/em590)	1,298 ± 244	3,885 ± 745**	2,012 ± 590	6,387 ± 396**,***	7,432 ± 244*	7,212 ± 112	6,984 ± 325*	7,188 ± 161
IL-1α (pg/ml)	15 ± 5	15 ± 5	6 ± 4	15 ± 8	14 ± 5.	175 ± 26**	20 ± 8	198 ± 25**
IL-2 (pg/ml)	13 ± 6	46 ± 22	20 ± 8	221 ± 55**	100 ± 23*	276 ± 112	75 ± 4	154 ± 65
IL-4 (pg/ml)	ND	66 ± 22	13 ± 4	85 ± 15**	18 ± 3	130 ± 10**	23 ± 8	141 ± 9**
IL-5 (pg/ml)	ND	ND	ND	17 ± 11	17 ± 11	102 ± 9	ND	123 ± 10
IL-6 (pg/ml)	148 ± 131	89 ± 26	44 ± 30	518 ± 155**,***	17 ± 7	627 ± 112**	33 ± 5	623 ± 147**
IL-10 (pg/ml)	ND	9 ± 4	ND	ND	ND	81 ± 12	ND	93 ± 12
IL-13 (pg/ml)	ND	77 ± 67	ND	1,505 ± 615***	591 ± 461	4,305 ± 678**	494 ± 261	4,067 ± 1,041**
IL-17 (pg/ml)	ND	46 ± 12	ND	524 ± 236	686 ± 657	1,833 ± 1,017	208 ± 105	1,840 ± 580
IL-21 (pg/ml)	ND	ND	ND	ND	ND	64 ± 15	ND	60 ± 17
IL-22 (pg/ml)	ND	45 ± 32	ND	54 ± 10	415 ± 323	1,713 ± 562**	538 ± 275	1,756 ± 243
IL-27 (pg/ml)	ND	ND	ND	ND	ND	394 ± 54	ND	444 ± 47
IFN-γ (pg/ml)	ND	83 ± 24	ND	699 ± 161**,***	120 ± 62	≥20,000**	279 ± 108	≥20,000**
TNF-α (pg/ml)	66 ± 24	110 ± 29	14 ± 5*($P = 0.06$)	160 ± 24**	191 ± 61	247 ± 30	274 ± 95*	288 ± 6
GM-CSF (pg/ml)	42 ± 26	61 ± 39	64 ± 26	243 ± 56**	122 ± 11	543 ± 74**	64 ± 37	513 ± 110**

Primary splenocytes were isolated from EAE mice treated with $SAgA_{PLP:LABL}$ or vehicle (PBS) during peak of disease (day 12) or disease remission (day 25). Splenocytes were stimulated with 25 µmol/l PLP or vehicle for 120 hours, and culture supernatant was collected. For cellular metabolism rates, cells were then incubated with resazurin (75 µmol/l final), and fluorescence (ex560/em590) was determined 24 hours later. Data are expressed as mean ± SEM, $n = 4$–6 mice per group.

*Indicates significance ($P \leq 0.05$) from untreated day 12 splenocytes treated with PBS *in vivo*. **Indicates significance ($P \leq 0.05$) from respective unstimulated splenocytes. ***Indicates significance ($P \leq 0.05$) from unstimulated EAE splenocytes isolated from vehicle treated mice during respective disease day.

EAE, experimental autoimmune encephalomyelitis; ex560/em590, $560_{excitation}/590_{emission}$; GM-CSF, granulocyte macrophage colony-stimulating factor; IFN, interferon; IL, interleukin; ND, not detectable; PBS, phosphate-buffered saline; PLP, proteolipid protein peptide; SAgA, soluble antigen array; $SAgA_{PLP:LABL}$, SAgA cografted with PLP and LABL peptides; TNF, tumor necrosis factor.

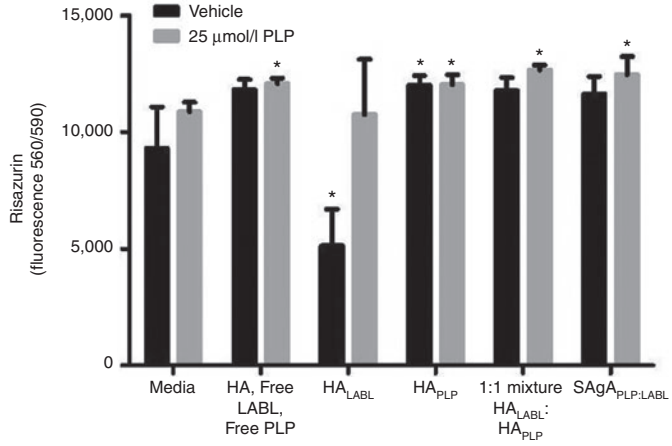

Figure 3 Cellular metabolism rates in experimental autoimmune encephalomyelitis (EAE) splenocytes harvested during disease remission (day 25) and retreated *ex vivo*. Primary splenocytes were isolated from EAE mice disease remission (day 25) and were treated *ex vivo* as indicated. Cells were then stimulated with 25 µmol/l proteolipid protein peptide (PLP; gray bars) or vehicle (media; black bars) for 120 hours and incubated with risazurin (75 µmol/l final). Fluorescence ($560_{excitation}$/$590_{emission}$) was determined 24 hours later ($n = 4$ mice per group). *Indicates significance ($P < 0.05$) from media treated, unstimulated (vehicle primed) splenocytes. HA, hyaluronic acid; HA$_{LABL}$, HA with grafted LABL peptide; HA$_{PLP}$, HA with grafted PLP peptide; PBS, phosphate-buffered saline; SAgA$_{PLP:LABL}$, soluble antigen array cografted with PLP and LABL peptides.

disease antigen, PLP, for treatment of EAE in mice, a murine model for MS.

In MS and animal models such as EAE, destruction of myelin in the central nervous system is propagated at least in part by CD4$^+$ T cells.[8,14,39] Current antigen-SITs focus on treating MS through modification of antigen via peptide constructs. Altered peptide ligands,[40–43] splenocytes coupled with myelin basic protein or PLP-derived peptides,[44,45] soluble PLP$_{139–151}$: major histocompatibility complex receptor construct,[46] and the FDA-approved synthetic copolymer glatiramer acetate (Copaxone (R)), look to generate a Th2 response and/or induce tolerance through "regulatory" T cells due to altered recognition of modified antigen.[47–49]

Alternatively, non–antigen-SITs seek to disrupt molecular signaling or cell adhesion events via monoclonal antibodies such as anti-CD28 Fab,[50] anti-CD40L antibody,[51] and the FDA-approved natalizumab marketed as TYSABRI (R) (biogen idec, Cambridge, MA), an anti-α4 integrin antibody.[52] By impeding a secondary "context" signal such as costimulation or immune cell adhesion, events such as the formation of the immunological synapse, the interface between professional antigen-presenting cells and T cells, can be prevented prohibiting the activation of CD4$^+$ T cells or lymphocyte recruitment.[23,53] Unfortunately, general inhibition of events such as cell adhesion can have unwanted and disastrous immunosuppressive effects.[14,52] While both antigen-SIT and secondary "context" signal interruption provide some therapeutic benefit, combining both the approaches could potentially enhance antigen-SIT efficacy, offering the specificity that is lacking in global disruption of secondary "context" signals.

Our previous studies found that HA conjugated to both PLP autoantigen and LABL inhibitory peptide (SAgA$_{PLP:LABL}$) suppressed EAE, but a mixture of unconjugated HA and free peptides did not, suggesting that conjugation of these peptides is required for autoimmune protection *in vivo*. The need for simultaneous delivery of both signals on the SAgA construct was further investigated by testing a 1:1 mixture of HA$_{LABL}$ and HA$_{PLP}$. The 1:1 mixture protected mice from EAE to an extent similar to that of SAgA$_{PLP:LABL}$; however, mice given equivalent doses of HA$_{LABL}$ or HA$_{PLP}$ independently did not improve disease outcomes in EAE mice. This result is strikingly similar to other approaches requiring conjugation for codelivery of antigen and immune inhibitor in other models of autoimmunity.[54–56]

Because the immune system is highly mobile, dynamic, and poorly understood, a tremendous challenge is present when trying to screen immune therapies. Therefore, we explored an *ex vivo* model using cellular viability and cytokine production as end points to systematically screen immunomodulatory compounds. Stimulated splenocytes from EAE mice were used to explore parameters such as signaling events initiated by SAgAs. Splenocytes collected at day 12 showed dramatic increase in cytokine expression levels of IL-2, IL-5, IL-6, IL-13, IL-22, TNF-α, and IFN-γ in PLP rechallenged splenocytes treated with SAgA$_{PLP:LABL}$ (Table 2). Although the cytokine expression was more robust at day 25 in response to PLP rechallenge, there was no difference in cytokine expression levels between SAgA$_{PLP:LABL}$ and PBS treatment (Table 2). This may suggest that many of the PLP-reactive splenocytes migrated out of the spleen by day 12 and that SAgA$_{PLP:LABL}$ may help "sequester" PLP-reactive cells within the spleen, thus limiting the potential pool of myelin-damaging lymphocytes in the periphery. Supporting this hypothesis is the qualitative observation that during peak of disease, EAE mice treated with PBS had extremely small spleens and splenocyte recovery was ~25% less compared with EAE mice treated with SAgA$_{PLP:LABL}$ or healthy matched control mice (data not shown). Alternatively, several of these cytokines (TNF-α, IFN-γ, and IL-6) have been shown to protect against EAE, suggesting that SAgA$_{PLP:LABL}$-dependent induction of these cytokines may be protective in this model; however, these cytokines have been shown to promote damaging inflammation in EAE studies.[57,58]

Several cytokines induced by PLP rechallenge were significantly reduced by various treatments involving the conjugation of HA:PLP:LABL. *Ex vivo* cytokine expression, however, did not correlate to *in vivo* clinical responses. When examined using *ex vivo* EAE splenocyte cultures, the component mixture (free PLP, free LABL, and free HA) had a similar cytokine expression profile as that of the conjugated form (SAgA$_{PLP:LABL}$). Perhaps a reason for this response is that the classical "2-signal" immune signaling is based on the presence of both antigen and "context" signal in the same time and space. This basic *ex vivo* screen may have a "containment" effect, placing both antigen and LABL in the same time and space artificially. If this assumption holds true, this may suggest that HA acts as a peptide "tether" *in vivo* to simply increase the local concentration of both peptides. Alternatively, covalent conjugation of PLP and LABL may deliver antigen and LABL above a threshold valency required to illicit an immune response.[59,60] Interestingly, similar *in vivo* protection was observed in mice treated with an equivalent dose of 1:1 HA$_{PLP}$:HA$_{LABL}$ mixture or SAgA$_{PLP:LABL}$; however, EAE splenocytes given these treatments had very different cytokine expression levels (Figure 4). Further studies are needed to identify the mechanisms whereby SAgA$_{PLP:LABL}$ and the 1:1 HA$_{LABL}$:HA$_{PLP}$ mixture protect mice against EAE.

Conclusion

In this study, we explored the mechanisms of SAgAs as novel antigen-SIT for the treatment of EAE, a murine model of MS, by combining delivery of antigen with delivery of a peptide known to inhibit immune cell adhesion. The *in vivo* data showed that

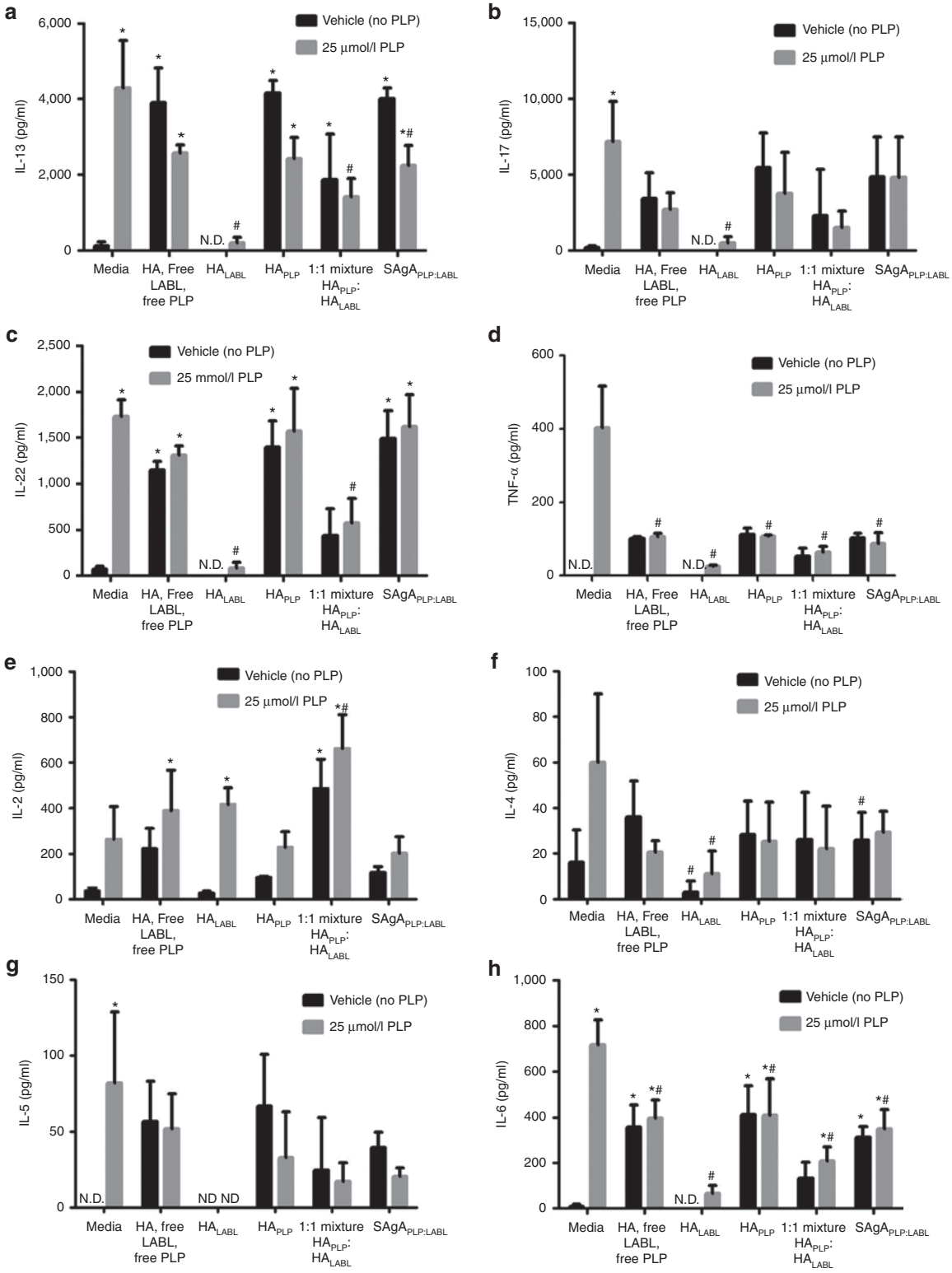

Figure 4 Primary splenocytes were isolated from experimental autoimmune encephalomyelitis (EAE) mice treated with phosphate-buffered saline (PBS; control group) at the end of study (day 25). Splenocytes were stimulated with 25 μmol/l proteolipid protein peptide (PLP; gray bars) or vehicle (media; black bars) and cotreated with component mixture (2.5 μmol/l hyaluronic acid (HA), 25 μmol/l free PLP peptide, and 25 μl free LABL peptide), 2.5 μmol/l HA with grafted LABL peptide (HA_{LABL}), 2.5 μmol/l , HA with grafted PLP peptide (HA_{PLP}), 1.25:1.25 μmol/l HA_{LABL}:HA_{PLP} mixture, or 2.5 μmol/l soluble antigen array cografted with PLP and LABL peptides ($SAgA_{PLP:LABL}$) for 120 hours. Levels of (**a**) Interleukin (IL)-2, (**b**) IL-4, (**c**) IL-5, (**d**) IL-6, (**e**) IL-13, (**f**) IL-17, (**g**) IL-22, and (**h**) tumor necrosis factor-α (TNF-α) in the cell culture supernatant were determined. Data are expressed as mean ± SD from splenocytes isolated from four independent mice ($n = 4$). *Indicates statistical significance ($P < 0.05$) from unstimulated splenocytes treated with vehicle (media). #Indicates statistical significance from stimulated splenocytes (25 μmol/l PLP) treated with vehicle (media). ND, not detectable.

codelivery through conjugation of both antigenic peptide (PLP) and an ICAM-1 inhibitor (LABL) to HA, either along a heteropolymer ($SAgA_{PLP:LABL}$) or an equivalent combined dose of two homopolymers (HA_{PLP} and HA_{LABL}), enhanced clinical score outcomes. Conversely, when tested *ex vivo* using splenocytes from EAE mice, cytokine profiles showed that as long as HA, PLP, and LABL were present in the sample, regardless of conjugation state, a response was generated. The *ex vivo* data showed a high dependence on the molecular characteristics of treatments while *in vivo*, the physics behind their copresentation was just as important.

These data confirm that in order to identify safer and more efficacious therapies to treat autoimmune diseases such as MS, we must understand the mechanisms whereby delivering a conjugated antigen and a secondary "context" signal therapeutic inhibits disease. Furthermore, because codelivery of both antigen and a secondary "context" signal through separate homopolymers was effective at treating EAE, this therapeutic paradigm may ultimately be adaptable as a supplemental additive formulation to current antigen only therapeutics such as Copaxone (R) (myelin antigen derivative) and therapeutics such as TYSABRI (R) (surface receptor) inhibitor. Antigen-SIT combinations with secondary "context" signals or inhibitors needs to be further investigated to improve our understanding of the individual contribution of each signal in altering autoimmune disease progression.

MATERIALS AND METHODS

Materials

HA, with an average molecular weight of 16.9 kDa, was purchased from Lifecore Biomedical (Chaska, MN). Analytical grade acetonitrile and synthesis grade trifluoro acetic acid were purchased from Fisher Scientific (Waltham, MA). Research grade sodium acetate, acetic acid, 7-hydroxy-3*H*-phenoxazin-3-one 10-oxide, and D_2O were purchased from Sigma (St. Louis, MO). Complete Freund's adjuvant and killed *Mycobacterium tuberculosis* strain H37RA were purchased from Difco (Sparks, MD). Water was provided by a Labconco (Kansas City, KS) Water PRO PS ultrapure water purification unit.

Peptide synthesis

Aminooxy peptides were synthesized using 9-fluorenylmethyloxycarbonyl–protected amino acid chemistry on polyethylene glycol-polystyrene resins. The peptides synthesized were aminooxy-LABL (aminooxy-ITDGEATDSG, *Ao-LABL*), a ligand of ICAM-1 and aminooxy-PLP (aminooxy–HSLGKWLGHPDKF, *Ao-PLP*), an antigen derived from proteolipid protein amino acids 139–151 (PLP137–151). Peptides were deprotected, cleaved from resin, and isolated by precipitation in ether. Purification was completed using preparatory HPLC followed by lyophilization. Peptide identity was verified, and purity/content was assessed using mass spectroscopy and analytical HPLC.

Reaction of aminooxy peptides to polymers

HA was dissolved in 20 mmol/l acetate buffer (pH 5.5 ± 0.1), and aminooxy reactive peptides were added. When both LABL and PLP peptides were used, each was weighed separately, dry powders were mixed, and then added simultaneously to reaction solution. After addition of the peptide(s), the reaction solution pH was adjusted back to 5.5 ± 0.1. Reaction solutions were stirred at 500 rpm using magnetic stir bars for ~16 hours. After the reaction, the SAgA ($SAgA_{PLP:LABL}$) or HA-conjugate control (HA_{PLP}, HA_{LABL}) products were purified by extensive dialysis to remove any unreacted peptide and were then lyophilized.

Gel permeation chromatography

The relative molecular weight of the HA and of the SAgAs was estimated using a Waters (Waters Corp., Milford, MA) 2695 Separation Module with column heater set at 25 °C, and samples were detected using Waters 2414 refractive index detector and Waters 2489 UV/Vis and injection volume of 50 µl. A tandem column setup of two Agilent (Agilent Technologies, Santa Clara, CA) Aquagel-OH 40 columns (Agilent) was used at a flow rate of 0.6 ml/minute with isocratic elution in 0.1 mol/l ammonium acetate buffer pH 5.0 with 0.25 mol/l NaCl for 45 minutes. Analysis was completed using Empower 3 software suite (Waters Corp., Milford, MA).

High-performance liquid chromatography

Quantification of free peptide postreaction was accomplished by gradient reversed phase HPLC (SHIMADZU, SHIMADZU Corp., Kyoto, KYT) using a Vydac HPLC protein and peptide C18 column. HPLC system was composed of an SCL-20A SHIMADZU system controller, LC-10AT VP SHIMADZU liquid chromatograph, SIL-10A XL SHIMADZU autoinjector set at 75 µl injection volume, DGU-14A SHIMADZU degasser, sample cooler, and SPD-10A SHIMADZU UV-vis detector (220 nm). A personal computer equipped with SHIMADZU class VP software controlled the HPLC-UV system. Gradient elution was conducted at a constant flow of 1 ml/minute, from 100% A to 35% A (corresponding to 0% B to 65% B) over 50 minutes, followed by an isocratic elution at 75% B for 3 minutes. Mobile phase compositions were (A) acetonitrile-water (5:95) with 0.1% trifluoro acetic acid and (B) acetonitrile-water (90:10, v/v) with 0.1% trifluoro acetic acid. At the completion of each analysis, the cartridge was equilibrated at initial conditions of 1 ml/minute flow rate for 5 minutes with A.

Induction of EAE and therapeutic study

SJL/J (H-2s) female mice, 4–6 weeks old, were purchased from Harlan Laboratories (Indianapolis, IN) and housed under specified, pathogen-free conditions at The University of Kansas (Lawrence, KS). All protocols involving live mice were approved by the Institutional Animal Care and Use Committee. Mice were immunized subcutaneously (s.c.) with 200 mg of PLP139–151 in a 0.2-ml emulsion composed of equal volumes of PBS and complete Freund's adjuvant containing killed *M. tuberculosis* strain H37RA (final concentration of 4 mg/ml; Difco). The PLP139–151/complete Freund's adjuvant was administered to regions above the shoulders and the flanks (total of four sites; 50 µl at each injection site). In addition, 200 ng/100 µl of pertussis toxin (List Biological Laboratories, Campbell, CA) was injected intraperitoneally (i.p.) on the day of immunization (day 0) and 2 days postimmunization. The mice received s.c. injections of each sample, equivalent to 200 nmol PLP/100 µl (HA_{LABL} 200 nmol), based on peptide concentration values calculated using HPLC, on days 4, 7, and 10. Disease progression was evaluated blindly by the same observer using clinical scoring as follows: 0, no clinical signs of the disease; 1, tail weakness or limp tail; 2, paraparesis (weakness or incomplete paralysis of one or two hind limbs); 3, paraplegia (complete paralysis of two hind limbs); 4, paraplegia with forelimb weakness or paralysis; and 5, moribund (mice were euthanized if they were found to be moribund). Body weight was also measured daily.

Splenocyte isolation and *ex vivo* treatment of splenocytes

Mouse spleens were resected 12 or 25 days postimmunization as experimentally appropriate, passed through a wire mesh using the rubber end of a sterile 1-ml syringe plunger, and collected in 5 ml of RPMI 1640 media. The crude cellular extract was then centrifuged at 1,100*g* for 5 minutes, and the resulting cell pellet was resuspended in 3.5 ml of 1× Gey's lysis solution and placed on ice for 3.5 minutes to lyse splenic red blood cells. The lysis reaction was stopped by the addition of 10.5 ml of RPMI 1640 media containing 10% fetal bovine serum and was centrifuged at 1,100*g* for 5 minutes. The remaining cell pellet was resuspended in fresh media (RPMI 1640 media containing 10% FBS and 1% penicillin–streptomycin) and seeded in 96-well cell culture plates at a cell density of 1×10^6 cells/well in a final volume of 100 µl. For *in vitro* rechallenge assays, splenocytes were then immediately stimulated with 25 µmol/l PLP peptide or vehicle (RPMI media). For select studies, vehicle- and PLP-stimulated splenocytes were also cotreated with 2.5 µmol/l HA_{LABL}, 2.5 µmol/l HA_{PLP}, a 1:1 mixture of 1.25 µmol/l HA_{LABL} and 1.25 µmol/l HA_{PLP}, 2.5 µmol/l $SAgA_{PLP:LABL}$, an equivalent mixture of noncovalently linked SAgA components (2.5 µmol/l HA, 25 µmol/l LABL, and 25 µmol/l PLP), or vehicle (RPMI media). Stimulated cell cultures were incubated for 120 hours at 37 °C in a CO_2 (5%) incubator.

Measurement of *in vitro* cytokines and cellular metabolism

Cell culture supernatant levels of cytokines were determined by a commercially available bead array ELISA kit and supporting Flow Cytomix Software (ebiosciences, San Diego, CA). Cell viability (metabolic capacity) was determined by a resazurin (7-hydroxy-3*H*-phenoxazin-3-one 10-oxide) assay.

Briefly, resazurin (75 µmol/l final) was added to splenocyte cultures and incubated for an additional 24 hours, and metabolic reductive capacity was determined by a change in fluorescence ($560_{excitation}/590_{emission}$). Background fluorescence was determined in sterile FBS, and resazurin containing RPMI media and was subtracted from each experimental read.

Statistical analysis

Statistical differences were determined by comparing treated groups with the negative control (PBS) for clinical disease score and body weight. Data were analyzed with a one- or two-way analysis of variance as experimentally appropriate followed by Fisher's least significant difference *post hoc* test. A P value <0.05 (or 0.01) was considered the threshold of statistical significance. All analyses were performed using GraphPad Software (GraphPad Software, La Jolla, CA).

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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