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A CD40 targeting peptide prevents severe symptoms in experimental autoimmune encephalomyelitis

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ABSTRACT

CD40/CD154-interaction is critical in the development of Experimental Autoimmune Encephalomyelitis (EAE; mouse model of Multiple Sclerosis). Culprit $CD4^+CD40^+$ T cells drive a more severe form of EAE than conventional CD4 T cells. Blocking CD40/CD154-interaction with CD154-antibody prevents or ameliorates disease but had thrombotic complications in clinical trials. We targeted CD40 using a CD154-sequence based peptide. Peptides in human therapeutics demonstrate good safety. A small peptide, $KGYY_6$, ameliorates EAE when given as pretreatment or at first symptoms. $KGYY_6$ binds Th40 and memory T cells, affecting expression of CD69 and IL-10 in the CD4 T cell compartment, ultimately hampering disease development.

1. Introduction

Multiple Sclerosis (MS) is an inflammatory demyelinating disease of the central nervous system (CNS). Rather than being only a disease of the CNS, MS is primarily an autoimmune disease and it is understood that several autoimmune components drive the disease. This aspect was elucidated primarily from studies in the Experimental Autoimmune Encephalomyelitis (EAE) mouse model and from analysis of lesions in brain and spinal cord of human patients. Sclerotic lesions form in the brain and spinal cord in both MS and EAE and involve infiltrating inflammatory cells, including macrophages (Baeten et al., 2008), mast cells (Hong et al., 2013), and T cells (El-behi et al., 2010; Fletcher et al., 2010; Howard et al., 1999). While $CD8^+$ T cells play a role in EAE (Huseby et al., 2001) and MS (Denic et al., 2013), $CD4^+$ T cells take center stage as drivers of the disease. Th1 cells, which produce IFNγ, TNFα, IL-1β and GM-CSF, are centrally involved in inflammation. IL-6 was originally described as a Th2, non-inflammatory cytokine, but is associated with inflammatory responses in MS (Furuzawa-Carballeda et al., 2007). Th17 cells that produce $IL\text{-}17_{A\text{-}F}$, IL-21 and IL-22, likewise are associated with EAE and MS. We recently demonstrated that $CD4^+CD40^+$ T cells (Th40), which are capable of producing both Th1 and Th17 cytokines simultaneously (Vaitaitis et al., 2017a), drive a more severe form of EAE than conventional $CD4^+$ T cells (Vaitaitis et al., 2017b).

CD40/CD154 interaction is crucial in EAE (Girvin et al., 2002, Vaitaitis, Yussman, 2017b). The classically described T cell co-stimulus is CD28 on T cells interacting with B7-1 or B7-2 on antigen presenting cells. Immunologic dogma holds that blocking CD28 interactions results in anergy, a total lack of response (Lenschow and Bluestone, 1993; Oliveira-dos-Santos et al., 1999). In EAE, the contrary was shown. Early studies suggested that CD28 co-stimulation was crucial for EAE development (Oliveira-dos-Santos et al., 1999) and in a later study, using $CD28^{-/-}$ mice, the initial disease induction challenge resulted in no disease (Girvin et al., 2002). However, a second challenge of the $CD28^{-/-}$ mice resulted in a much more severe, fulminant disease (Girvin et al., 2002). The treatment that did prevent disease under these conditions was CD40/CD154 block(Girvin et al., 2002). The finding suggests CD40 as a central nexus for disease onset and severity, and further suggests that CD40 expression on T cells, as an inflammatory co-stimulus, is the crucial factor for disease development. Preventing the interaction of CD40 and CD154 in mice by using blocking antibodies decreases the severity and delays onset of EAE (Girvin et al., 2002; Hong et al., 2013) and similar beneficial results from blocking CD40/CD154 interaction occur in other autoimmune disease models (Toubi and Shoenfeld, 2004; Waid et al., 2004).

A problem area however, is that when attempting to block CD40/CD154 interaction with anti-CD154 antibody in human trials, severe clinical problems often arise. In human Systemic Lupus Erythematosus, unfortunate life-threatening thromboembolic events occurred when using anti-CD154 antibodies in clinical trials (Boumpas et al., 2003; Sidiropoulos and Boumpas, 2004; Toubi and Shoenfeld, 2004) and the studies were halted. Antibodies as therapeutics come with inherent

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problems in that they may be immunogenic in the recipient and they can bind to Fc-receptors on cells such as platelets, neutrophils and macrophages, increasing risk of anaphylaxis. In the case of CD154 antibodies, CD154 interacts with the αIIbβ3 integrin on platelets, which stabilizes thrombi and it is therefore likely that the CD154 antibody blocked that interaction, destabilizing the thrombi. Because of these problems, a better way of blocking CD40/CD154 interactions is necessary. To that end, F(ab) fragments of CD154 antibodies, lacking the Fc-domain, are being pursued (Shock et al., 2015). However, the fragments still are bulky molecules that can be immunogenic. It is also possible that bulky molecules are sterically hindered to contact their targets in a "crowded" situation such as in an immunological synapse. Other strategies include random peptides that bind to CD40 in-vitro (Kitagawa et al., 2005) but those also bind other targets in-vivo, and small organic molecules that also bind CD40 but lose their activity in protein-rich medium (Margolles-Clark et al., 2009b). Additionally, those small organic molecules are highly similar to suramin, which has toxic side effects (Kaur et al., 2002; Margolles-Clark et al., 2009a).

Given the aforementioned problems, we developed a more targeted approach to inhibit the CD40/CD154 interaction. Based on the protein domain of CD154 that is essential for interaction with CD40, we designed several peptides that span the amino acids known to be critical (Bajorath et al., 1995a; Bajorath et al., 1995b; Vaitaitis et al., 2014). In the non-obese diabetic (NOD) mouse model of Type 1 Diabetes (T1D), one particular 15-mer peptide was highly efficacious in preventing diabetes onset and even reversed hyperglycemia in 56% of newly hyperglycemic mice (Vaitaitis et al., 2014). The CD154 sequence spanned by this peptide is 80% identical between the mouse and human sequences (S1Fig), suggesting that it is functionally important. Here we are utilizing a smaller, core region, of the 15-mer, a 6 amino acid sequence (S1Fig; referred to as $KGYY_6$), to prevent or ameliorate EAE symptoms, in the mouse model for MS. The $KGYY_6$ peptide sequence has 83% homology between mouse and human, and the 3 amino acids that are critical for interaction with CD40 are 100% conserved. Peptides are often used in human therapeutics and so far demonstrate a good safety record (Hinke, 2008; Johnson, 2012; Moertl et al., 2011).

2. Materials and methods

2.1. Mice

C57BL/6 mice were from Taconic (Hudson, NY, USA). B6.CD40KO mice were from The Jackson Laboratory (Bar Harbor, ME, USA). Animals were housed at the University of Colorado Denver Anschutz Medical Campus AALAC approved Vivarium. This study was carried out in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. The protocol was approved by the Institutional Animal Care and Use Committee at the University of Colorado Denver (Protocol number: B-55817(01)1E). All efforts were made to minimize suffering and euthanasia was performed using carbon-dioxide followed by either cervical dislocation or exsanguination.

2.2. Antibodies and reagents

For flow cytometry: Anti-IFNγ (XMG1.2), anti-IL17A (eBio1787), anti-TNFα (MP6-XT22), anti-IL-10 (JES5-16E3), anti-IL-2 (JES6-5H4), anti-CD3 (145.2C11), anti-CD62L (MEL-14), anti-CD44 (IM7), anti-CD69 (H1.2F3), appropriate isotype control antibodies, and permeabilization buffer for intracellular stains were from eBioscience, Inc. (San Diego, CA, USA). Anti-CD4 (H129.19) was from BioLegend (San Diego, CA, USA). For cell purification: Lympholyte-M and Lympholyte-Mammal were from CedarLane (Burlington, NC, USA). Anti-CD40, 1C10 [22], was produced in-house. For EAE induction: M. Tuberculosis H37 RA was from Becton Dickinson and Company (Franklin Lakes, NJ, USA). Pertussis toxin (PT) was from Sigma-Aldrich® (St. Louis, MO, USA).

2.3. Induction of EAE

The EAE model can be highly variable even when controlling all possible parameters. This is likely due to seasonal variations as well as litter differences. In order to minimize litter-to-litter and seasonal variations, we performed large experiments that had 10 mice per group and randomly mixed the mice once they arrived from the vendor (this is possible with female mice that generally do not show aggression toward new mice). Female 10–12 week old C57BL/6 mice were immunized subcutaneously on the upper back/neck with 100 µl completely emulsified $MOG_{35–55}$ peptide (50 µg in 50 µl PBS) and complete Freund's adjuvant (CFA; 75 µg M. Tuberculosis H37 RA in 50 µl incomplete Freund's adjuvant (mineral oil)), followed by pertussis toxin (PT; 200 ng in 100 µl PBS) injections as described (Miller et al., 2010). Mice were then randomly assigned to a treatment cohort or vehicle control cohort. One cohort of mice received i.v. injections of a 6-mer peptide (AKKGYY; referred to as $KGYY_6$; 25 µg in 100 µl PBS per mouse) on days -3, -1, 6, 11, 13, and 15, with day 0 being the day of EAE induction. Another cohort received peptide on days -3, -1, 13, 20, and 27. Yet another cohort received peptide at the start of visible symptoms and two more times, days 11, 13, and 15, respectively. Vehicle control mice received injections of PBS. All mice were monitored daily for disease and scored: 0 – No abnormalities; 0.5 – Clutching hind limbs; 1 – Limp tail or weak hind limbs and/or wobbly gait; 1.5 – Limp tail and clutching hind limbs; 2 – Limp tail and weak hind limbs and/or wobbly gait. Mouse supports and propels itself using hind limbs; 2.5 – Limp tail and weak hind limbs and/or wobbly gait. Mouse cannot support and propel itself using hind limbs but the paws are moving; 3 – Limp tail and one weak hind limb, while the other is completely paralyzed. Mouse still uses the weak hind limb to propel itself somewhat; 3.5 – Limp tail and one weak hind limb, while the other is completely paralyzed. Mouse does not use the weak limb, which is almost at paralysis; 4 – Limp tail and complete hind limb paralysis. Often the mouse displays spastic hypertonia and involuntarily crosses its hind limbs; 5 – Complete paralysis of hind quarter and weak fore limb(s). The data are reported as the mean daily clinical score for all animals in each group. Mice reaching a level 5 or losing > 20% of their bodyweight were euthanized.

2.4. Flow cytometry

Peripheral blood mononuclear cells (PBMC), draining lymph nodes (dLN; cervical), and spleens were processed over Lympholyte (Cedarlane, Hornby, Ontario, Canada) according to manufacturer's protocol to generate purified lymphocytes. Cells were stained for extracellular proteins, washed and fixed in paraformaldehyde, then permeabilized and stained for intracellular proteins. Gates were set from appropriate isotype controls. Samples were run on a MACSQuant flow cytometer (Miltenyi Biotec Inc., Auburn, CA, USA). Data was analyzed with FlowJo software (FlowJo LLC, Ashland, OR, USA).

3. Results

3.1. $KGYY_6$ ameliorates the disease course of EAE

$KGYY_6$ comprises the core region of the CD154 protein and contains 3 of the amino acids required for interaction with CD40 (Bajorath, Chalupny, 1995a, Bajorath, Marken, 1995b). In previous work we tested a 15-mer version of the peptide, $KGYY_{15}$, which prevented diabetes onset in NOD mice, and reversed hyperglycemia in 56% of newly onset diabetic mice (Vaitaitis et al., 2014). The 15-mer has 80% homology between mouse and human (S1Fig). The $KGYY_6$ peptide has 83% homology between mouse and human and the amino acids known to be critical for interaction with CD40 are 100% conserved (S1Fig). We chose to test the 6-mer in a model of multiple sclerosis (EAE).

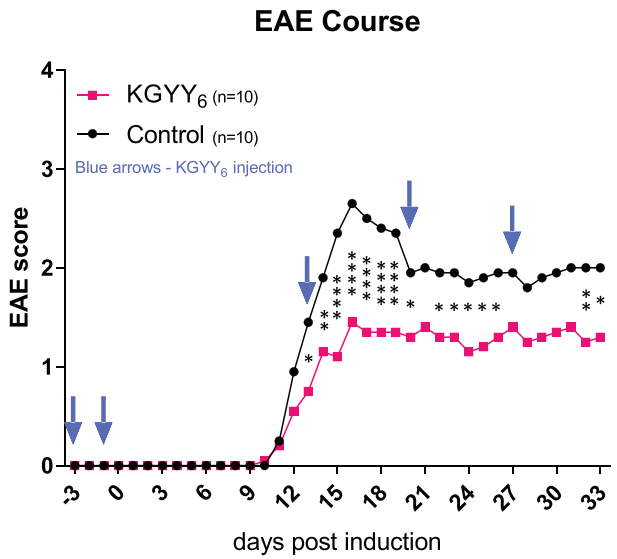

Fig. 1. $KGYY_6$ ameliorates EAE. C57BL/6 mice were either pretreated, or not, with $KGYY_6$ peptide then EAE was induced. $KGYY_6$ pretreated mice received boosters of $KGYY_6$ on days 13, 20, and 27 (blue arrows). Mice were disease scored daily. Statistical significance was calculated by Two-Way ANOVA with Sidak's multiple comparison test (asterisks denote p-value; * - < 0.05, **- < 0.01, *** - < 0.001, **** - < 0.0001). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

One group of mice received $KGYY_6$ i.v. injections 3 and 1 day(s) prior to EAE induction while another group received vehicle PBS. EAE was then induced and the mice started to show symptoms on day 10, which is normal for our laboratory (Fig. 1). On days 13, 20 and 27, the $KGYY_6$ group received a boost of peptide while the other group again received vehicle PBS. The disease course was significantly less severe in the $KGYY_6$ treated group compared to the control group (Fig. 1; $p < .0001$; two-way ANOVA). Already on day 13 there was a statistically significant difference that lasted throughout the experiment (Fig. 1; asterisks denote p-value; * - < 0.05, **- < 0.01, *** - < 0.001, **** - < 0.0001; two-way ANOVA with Sidak's multiple comparisons test).

3.2. Starting treatment at first sign of symptoms ameliorates the EAE disease course

In humans, it is not possible to know who is going to develop MS and, therefore, we addressed whether starting treatments at early signs of EAE would ameliorate symptoms in the test model. In our laboratory, disease symptoms typically start on day 10 or 11 post disease induction challenge. We treated a group of EAE induced mice starting on day 11 and then on days 13 and 15. We pretreated another group at days -3 and -1, and then further treated on days 6, 11, 13, and 15. Yet another group served as control for disease induction and received no treatment. The control group demonstrated a disease course identical to the previous experiment while the pretreated mice, again, had a significantly less severe disease course (Fig. 2; $p < .0001$; Two-way ANOVA with Tukey's multiple comparison test). The treat-at-symptom group also demonstrated a less severe disease course compared to control but was more severe than the pretreated group (Fig. 2; $p < .0001$ for both comparisons; Two-way ANOVA with Tukey's multiple comparison test).

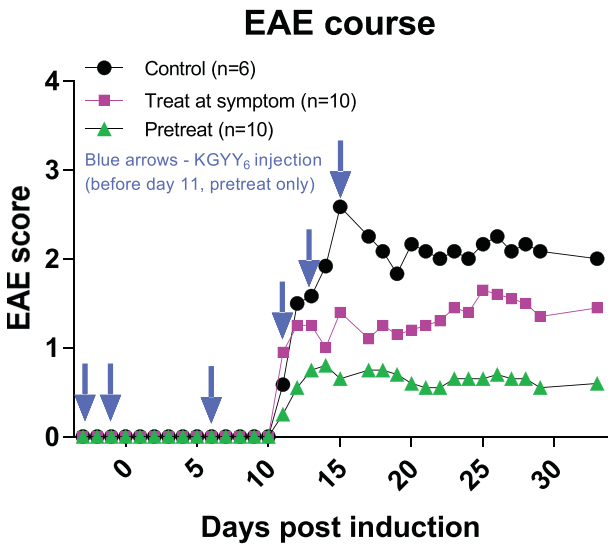

Fig. 2. Starting treatment at first symptoms ameliorates EAE. C57BL/6 mice were either pretreated at days −3 and −1, or not, with $KGYY_6$ peptide then EAE was induced. $KGYY_6$ pretreated mice received boosters of $KGYY_6$ on days 6, 11, 13, and 15 (blue arrows). One group, that was not pretreated, received $KGYY_6$ peptide at first symptom on day 11 as well as boosters on days 13 and 15. Mice were disease scored daily. Statistical significance was calculated by Two-Way ANOVAwith Tukey multiple comparison test. Control vs. Treat at symptom, Control vs. Pretreat, and Treat at symptom vs. Pretreat were all significantly different; $p < .0001$. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

3.3. Earlier boosts with $KGYY_6$ have a better impact on the EAE disease course

The disease course in the pretreated group in Fig. 1 appeared to be more severe than the disease course of the pretreated group in Fig. 2. The difference between the two was the timing of the peptide boosts that were administered after the initial pretreatment at days -3 and -1. When comparing the disease courses of the groups with the different boost schedules, clearly there was a significant difference in disease severity, with overall less severe disease if the boosts started early, at 6 days, rather than at 13 days (Fig. 3; < 0.0001; Two-way ANOVA with Tukey's multiple comparison test). In fact, administering the boosts later, at days 13, 20, and 27, caused a disease course similar to that of the treat-at-symptom group in Fig. 2.

3.4. T cells from $KGYY_6$ treated mice express more CD69

CD69 expression traditionally is described as an early or very early activation marker on T cells and is induced by TCR recognition of antigen and subsequent CD3 stimulation (Cibrian and Sanchez-Madrid, 2017). However, studies that are more recent have focused on the function of the molecule. CD69 is a membrane-bound, type II, C-lectin receptor that interacts with galectin-1 as a trans-ligand, and interacts with sphingosine-1-phosphate receptor (S1P1R) as a cis-ligand (Cibrian and Sanchez-Madrid, 2017). S1P1 promotes egress of T cells from lymph nodes and from tissues (Mackay et al., 2015) and CD69 interaction with S1P1R prolongs tissue retention. This is particularly relevant given that a S1P1R super-agonist, Gilenya, is one of the disease modulating therapies that is FDA approved for MS (Mao-Draayer et al., 2017). Gilenya's mechanism of action is to bind S1P1R and to down regulate its surface expression, which then retards T cells in lymph nodes. Based on the above, CD69 expression on cells from lymph nodes indicates cells poised for nodal retention. We determined the number of CD69 expressing cells in draining lymph nodes (dLN) and spleens

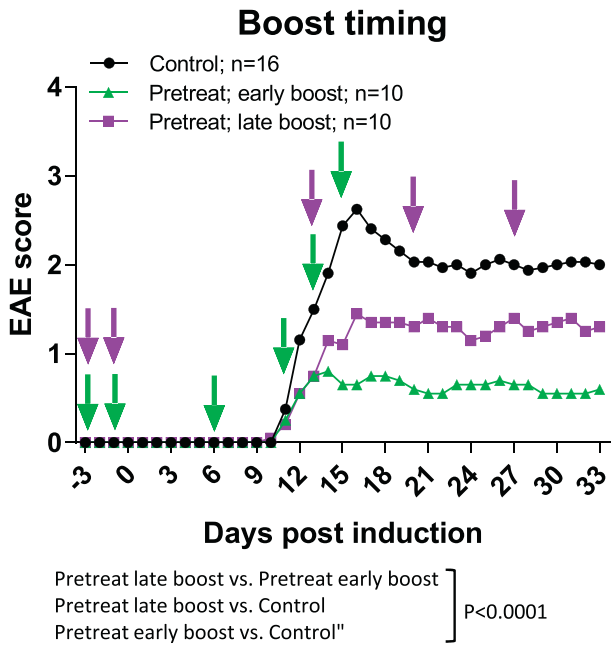

Fig. 3. Earlier boosts with $KGYY_6$ have a better impact on the EAE disease course. C57BL/6 mice were either pretreated, or not, with $KGYY_6$ peptide then EAE was induced. $KGYY_6$ pretreated mice received boosters of $KGYY_6$ either on days 6, 11, 13, and 15 (green arrows) or on days 13, 20, and 27 (purple arrows). Mice were disease scored daily. Statistical significance was calculated by Two-Way ANOVAwith Tukey multiple comparison test. Control vs. Treat at symptom, Control vs. Pretreat, and Treat at symptom vs. Pretreat were all significantly different; $p < .0001$. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

harvested from the EAE mice at the end of the experiment, after 33 days. The number of Th40 cells in dLN of treated mice expressing CD69 was increased when compared to vehicle treated controls, but the difference did not yet achieve significance (Fig. 4A; top left panel). However, one sample in the vehicle treated controls was an outlier and, if it was not taken into consideration, there was a significant difference ($p = .0387$, *t*-test). A significantly greater number of Th40 cells from spleens of $KGYY_6$ treated mice expressed CD69 compared to untreated mice (Fig. 4A; top right panel; $p = .0139$; *t*-test). Overall, fewer of the conventional CD4 T cells from dLN and spleen expressed CD69 compared to Th40 cells (Fig. 4A), but the number of conventional CD4 T cells expressing CD69 was increased by $KGYY_6$ treatment compared to the same cells from controls (Fig. 4A; bottom panels; $p = .0021$ (dLN) and $p = .0450$ (spleen); *t*-test). We considered CD69 expression on a per cell basis and found that in Th40 cells there was an increase in the amount of CD69 expressed per cell (Fig. 4B). As in Fig. 4A, the increase did not reach significance in the dLN, however, when the same outlier was taken out of consideration, there was a significant difference ($p = .0400$; *t*-test). Th40 cells from spleens of treated mice expressed significantly more CD69 per cell than the same cells from vehicle controls (Fig. 4B; right panel; $p = .0452$; *t*-test). While the percentage of conventional CD4 T cells that expressed CD69 was increased by $KGYY_6$ treatment, that treatment did not change the amount of CD69 per cell in this cell type (Fig. 4B; bottom panels).

L-selectins are expressed on T cells and, depending upon activation status, expression rapidly cycles between high and low levels, thus allowing cells to crawl to areas of inflammation, and to extravasate into the tissue (Butcher and Picker, 1996) (Chao et al., 1997). CD62L is an L-selectin that has generally been associated with naïve T cells, given its importance in lymph node retention (Kansas, 1992; Tedder et al., 1993). Rather than considering CD62L as a biomarker for naïve T cells, we considered that it may be involved in lymph node retention of potentially pathogenic effector cells. We examined the number of CD62L expressing Th40 and conventional CD4 T cells from both spleens and dLN of $KGYY_6$ treated mice and compared to vehicle treated mice. There was no difference in number of CD62L expressing cells between treated and untreated (Fig. 4C).

3.5. $KGYY_6$ treatment alters IL-10 production by conventional CD4 T cells

We examined intracellular cytokine expression profiles in Th40 and conventional CD4 T cells immediately ex-vivo at the end of the experiment, after 33 days. In conventional CD4 T cells from dLN, but not spleen, of $KGYY_6$ treated animals there was an increase in intracellular IL-10 compared to the same cells from untreated animals (Fig. 5; $p = .0145$; *t*-test). In Th40 cells, there was no differences in intracellular IL-10 expression between $KGYY_6$ treated and untreated mice in dLN or spleen (Fig. 5). Other cytokines (TNFα, IFNγ, IL-2, and IL-17A) were unchanged in response to $KGYY_6$ treatment in both cell types (Fig. S2).

3.6. $KGYY_6$ treatment does not affect memory cell levels

In previous work, we showed that memory cell levels increase in response to induction of EAE. Therefore, we determined whether treatment with $KGYY_6$ had an effect on memory cell levels. When examining the expression of $CD44^+CD62L^-$ (effector memory) and $CD44^+CD62L^+$ (central memory) in both spleens and dLN there was no difference in the levels of those memory phenotypes between treated and untreated Th40 or conventional CD4 T cells (Fig. S3A and B).

3.7. $KGYY_6$ peptide binds to EAE induced Th40 cells

To confirm that $KGYY_6$ binds to the intended target cells we harvested peripheral blood mononuclear cells (PBMC), dLN lymphocytes, and splenic lymphocytes from mice at 6 and 13 days out from receiving the full EAE regimen and stained them with a FITC-conjugated $KGYY_6$. Clearly, the peptide stained the $CD4^+CD40^+$ cells but not the $CD40^-$ cells in PBMC, dLN, and spleen samples (Fig. 6A, left panels). When gating on memory cells (CD62L versus CD44), the peptide stained primarily effector ($CD44^+CD62L^-$) and central ($CD44^+CD62L^+$) memory cells in PBMC and spleen samples but had a more diffuse pattern in dLN samples (Fig. 6A, right panels).

To confirm that the $KGYY_6$ peptide binds specifically to CD40, we stained PBMC from C57BL/6 mice that have the CD154-interacting domain of CD40 knocked out (B6.CD40KO). Clearly, the peptide did not stain the B6.CD40KO cells while it readily stained both the non-induced and EAE-induced C57BL/6 mice (Fig. 6B), demonstrating the specificity of the peptide for CD40.

4. Discussion

Early and ongoing treatment with disease-modifying therapy is currently the best care in relapsing remitting MS. Such treatments can reduce the number of relapses, delay progression of disability, and limit new lesions as seen on MRI. When severe relapses occur, corticosteroids are used to manage and reduce the inflammation in the CNS. Many of the current treatments for MS often have the side effect of broadly suppressing the immune system. While that may decrease disease symptoms, it also leads to an increased susceptibility to opportunistic infections. Therefore, the search for treatments that have a more targeted effect on the actual culprit immune cells is ongoing.

We recently demonstrated that Th40 cells drive a more severe form of EAE than conventional CD4 T cells do (Vaitaitis et al., 2017b). Th40 cells express the alternative costimulatory molecule CD40 (Baker et al., 2008; Munroe and Bishop, 2007), which is also expressed by other immune cells and is known to be a major target in autoimmune diseases

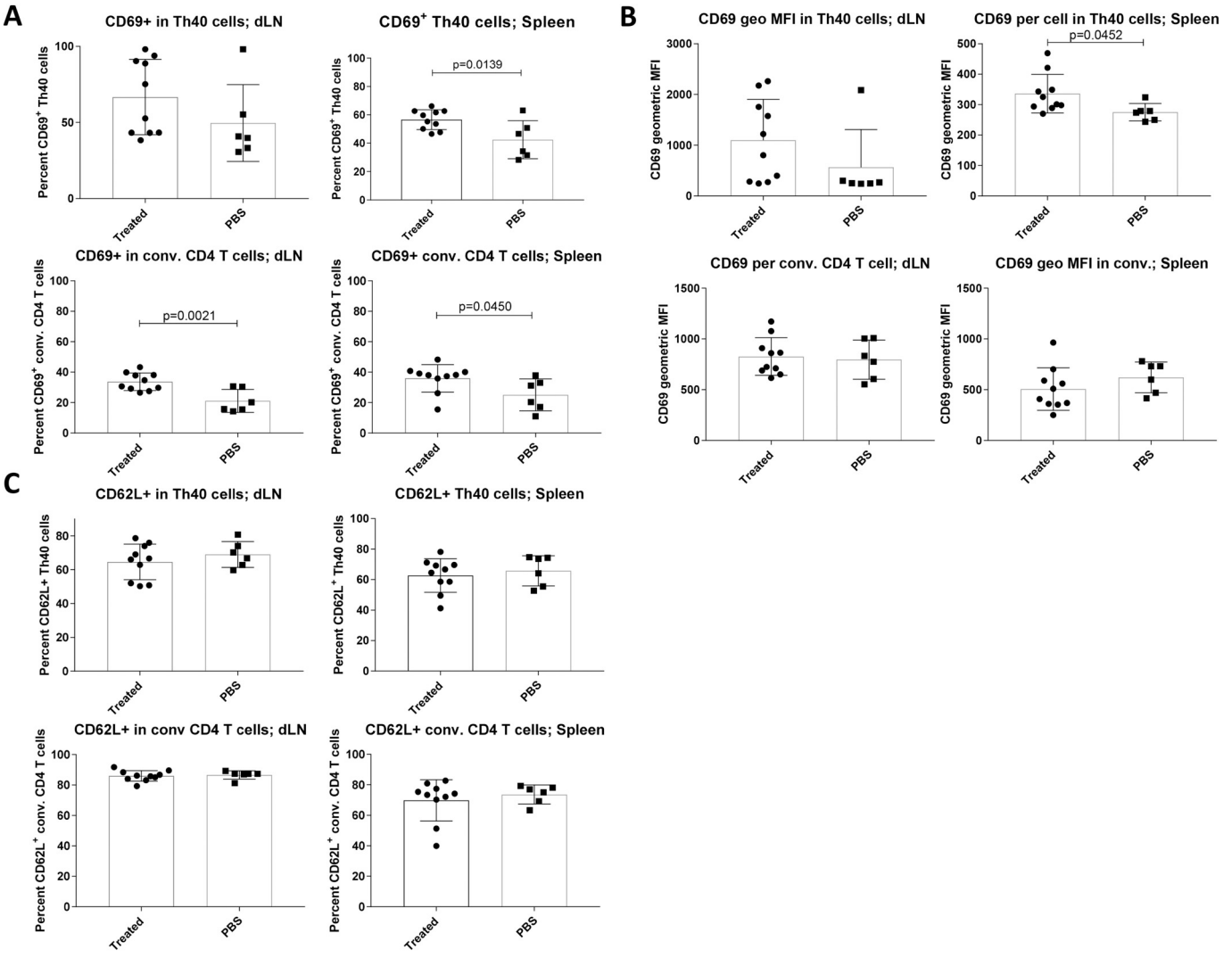

Fig. 4. T cells from $KGYY_6$ treated mice express more CD69. Lymphocytes from dLN or spleen were stained and gated on Th40 or conventional CD4 T cells then (A, B) CD69 and (C) CD62L expression was analyzed. Significance was calculated by *t*-test and p-values are indicated in the figure and the text.

(Peters et al., 2009). Interestingly, there appears to be several different forms of CD40 receptor, either because of glycosylation differences in the protein itself (Vaitaitis and Wagner Jr., 2010) or because of the interaction of CD40 with other molecules (Vaitaitis and Wagner Jr., 2010, 2012, 2013) or both. Certainly, a less glycosylated form of CD40 appears to be associated with more activated cells (Vaitaitis and Wagner Jr., 2010). Therefore, it would be ideal to target such activated immune cells specifically. It is known that EAE symptoms can be reduced by anti-CD154 antibody injections, which block CD40 signaling (Girvin et al., 2002), but anti-CD154 antibody treatments had serious and life-threatening side effects in clinical trials (Boumpas et al., 2003; Sidiropoulos and Boumpas, 2004; Toubi and Shoenfeld, 2004). Therefore, we set out to find a different, yet specific way of targeting CD40-signals. We previously demonstrated that a CD40-targeting peptide binds to CD40 and prevents and even reverses T1D in the NOD mouse model (Vaitaitis et al., 2014). This peptide avoids many of the problems that are inherent to antibodies, e.g. immunogenicity and anaphylaxis causality, and patients generally tolerate peptide treatments well.

Here we used a 6-mer peptide, which is predicted to be stable for at least 4.4 h (calculated by the ExPasy ProtParam tool) and spans the core region of the domain in the CD154 protein that interacts with CD40, to affect CD40 signals during the EAE disease process. Clearly, administration of the peptide ameliorated the symptoms whether pretreated then boosted or just treated at the first sign of symptoms. Pretreatment followed by boosts worked better than just treating at signs of symptoms. Obviously, by treating at the first signs of symptoms, we are trying to approximate human disease where it is desirable to treat only when disease symptoms onset rather than continually administering therapy. The limitation with the EAE studies is that onset can be gaged only by visual clues such as a limp tail, wobbly gait, paralysis etc. In human disease, an MS patient who is experiencing a relapse may very well be aware of telltale signs much earlier than the appearance of actual disability. Peptide therapy could therefore commence at much earlier stages, possibly having a greater impact. In the EAE experiments here, there was certainly a significantly better outcome if the peptide boosts were administered earlier, before any visible signs of disease. It would also be possible to keep those with diagnosed MS who are in remission in treatment with periodic peptide doses and then increase the dosing frequency if the patient reports early signs of a relapse.

Interestingly, treating EAE mice with $KGYY_6$ induced a significant increase in CD69 expression in dLN and spleen CD4 T cells (Th40 and conventional CD4 T cells), which recently was shown to prolong tissue retention of T cells (Cibrian and Sanchez-Madrid, 2017; Mackay et al., 2015). Also recently, circadian oscillations of lymphocytes trafficking to/from the vascular space and tissues/lymph nodes were demonstrated to have a significant effect on EAE disease severity, where EAE induction during the daytime caused more severe symptoms than if done 1 h after the start of the dark cycle (Druzd et al., 2017). We have shown

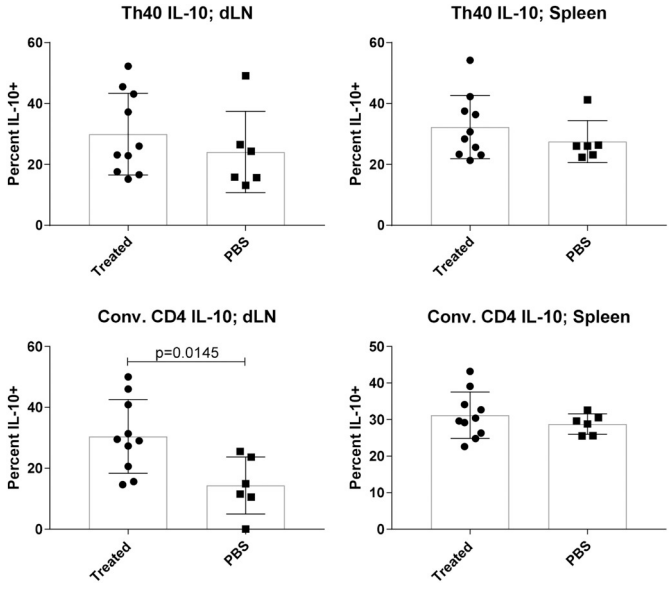

Fig. 5. $KGYY_6$ treatment alters IL-10 production by conventional CD4 T cells. Lymphocytes from dLN or spleen were stained and gated on Th40 or conventional CD4 T cells then intracellular IL-10 levels were analyzed. Significance was calculated by t-test and p-values are indicated in the figure and the text.

previously that CD40-signaling influences trafficking of leukocytes (Vaitaitis et al., 2017a). While not tested here, we speculate that the $KGYY_6$ treatment may affect the timely trafficking of culprit cells to the CNS for optimal disease induction. Previous data led us to the same speculation about timely trafficking (Vaitaitis et al., 2017b). It will be highly important to understand whether alterations in trafficking is a mechanism in the $KGYY_6$ treatment and whether time of day of administration impacts the efficacy.

$KGYY_6$ treatment induced an increase in IL-10 expression in conventional CD4 T cells from dLN samples. While IL-10 is considered anti-inflammatory, it is not clear from the current data whether the cells expressing IL-10 are exerting such an effect. Contrary to what we previously demonstrated when a similar peptide was used to prevent T1D (Vaitaitis et al., 2014), $KGYY_6$ did not cause a prevention of expansion in the Th40 cell population. NOD mice spontaneously develop T1D while EAE has to be induced in mice. It is unclear if the rather robust immune stimulation employed to induce EAE is simply to strong and overcomes an “expansion-prevention” aspect of the peptide treatment. In any case, $KGYY_6$ appears to directly target the culprit cells as we could demonstrate binding specifically to Th40 cells from EAE induced mice, the cells that we previously showed were causing a more severe disease in this model (Vaitaitis et al., 2017b). However, other cells express CD40 and inflammatory conditions can induce CD40 expression on previously surface-CD40-negative cells (Vaitaitis et al., 2017b). Therefore, it is likely that B cells, macrophages, and other immune cells would also bind the peptide at certain points throughout the disease

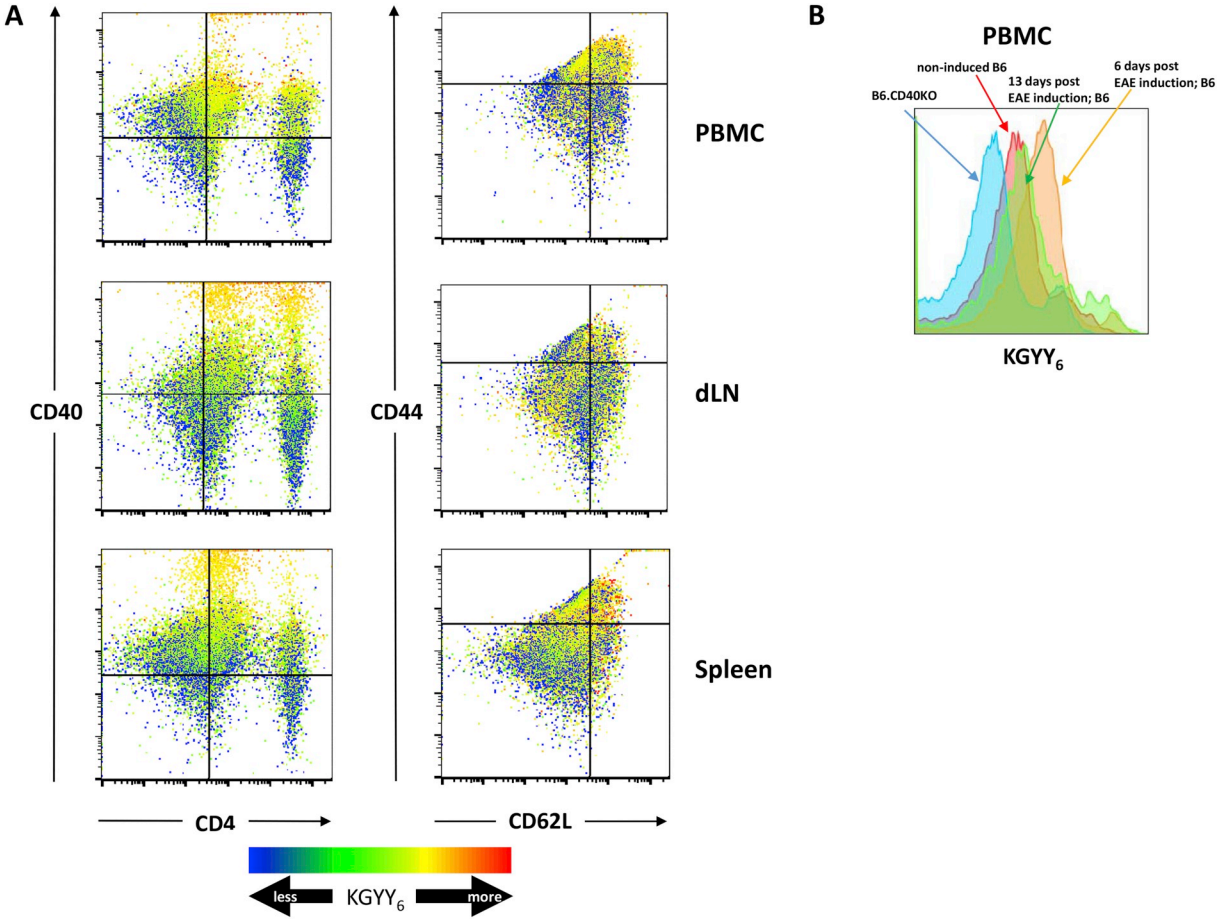

Fig. 6. KGYY6 binds Th40 and memory cells. C57BL/6 mice were EAE induced then PBMC, dLN, and splenic lymphocytes were harvested on day 6 or 13 and stained. (A) Dot plots on the left are CD4 vs. CD40 with heat-mapping for $KGYY_6$. Dot plots on the right are CD62L vs. CD44 with heat-mapping for $KGYY_6$. The more yellow and orange, the more staining for $KGYY_6$. The results shown are from the 6 days post EAE induction. (B) Histogram depicting KGYY6 staining of B6.CD40KO, control non-induced C57BL/6, and EAE-induced C57BL/6 mice 6 and 13 days post induction. Data represents 2 mice at 6 days and 3 mice at 13 days, all with similar results. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

course. In future studies, it will be important to assess the effect of $KGYY_6$ on those cells as well as target cells in the CNS, such as microglia, during EAE.

Whether the target cells are only Th40 cells or also other CD40 expressing cells the peptide does not eliminate or depress all T cells or even all Th40 cells. Therefore, it is likely that the $KGYY_6$ peptide would not be generally immunosuppressive. However, it will be important to confirm that that is the case. If so, $KGYY_6$ therapy would be advantageous compared to many current MS drug therapies that often immune suppress the patients.

Future large studies will be necessary to elucidate the exact mechanism(s) of action of the $KGYY_6$ peptide. Currently it is in toxicity and kinetics studies in mice with the ultimate aim of filing an Investigational New Drug application with the Food and Drug Administration such that clinical testing can commence.

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Author contributions

GMV designed and performed the experiments, analyzed the data, and wrote the manuscript. MGY performed experiments and critically reviewed the manuscript. DHW conceived of the peptide and critically reviewed the manuscript.

Competing interests

DMW is the co-founder and co-owner of Op-T LLC. MGY is the chief medical officer of Op-T LLC. GMV performs consultant work for Op-T LLC.

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