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Effects of a gut-selective integrin-targeted therapy in male mice exposed to early immune activation, a model for the study of autism spectrum disorder

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ABSTRACT

To clarify the role of gut mucosal immunity in ASD, we evaluated, in the early-life immune activation (EIA) mouse model, the effects of administration of a monoclonal antibody directed against the integrin alpha4 beta7 (α4β7 mAb), blocking the leukocyte homing into the gut mucosa. EIA is a double-hit variant of the maternal immune-activation (MIA) model, including both prenatal (Poly I:C) and postnatal (LPS) immune challenges.

In C57BL6/J EIA male adult offspring mice, IL-1β and IL-17A mRNA colonic tissue content increased when compared with controls. Cytofluorimetric analyses of lymphocytes isolated from mesenteric lymph-nodes (MLN) and spleens of EIA mice show increased percentage of total and $CD4^+\alpha4\beta7^+$, unstimulated and stimulated IL-$17A^+$ and stimulated IFN-γ^+ lymphocytes in MLN and $CD4^+\alpha4\beta7^+$ unstimulated and stimulated IL-$17A^+$ and stimulated IFN-γ^+ lymphocytes in the spleen. Treatment with anti-α4β7 mAb in EIA male mice was associated with colonic tissue IL-1β, and IL-17A mRNA content and percentage of $CD4^+$ IL-$17A^+$ and IFN-γ^+ lymphocytes in MLN and spleens comparable to control mice. The anti-α4β7 mAb treatment rescue social novelty deficit showed in the three-chamber test by EIA male mice. Increased levels of IL-6 and IL-1β and decreased CD68 and TGF-β mRNAs were also observed in hippocampus and prefrontal cortex of EIA male mice together with a reduction of BDNF mRNA levels in all brain regions examined. Anti-α4β7 mAb treatment restored the expression of BDNF, TGF-β and CD68 in hippocampus and prefrontal cortex.

Improvement of the gut inflammatory status, obtained by a pharmacological agent acting exclusively at gut level, ameliorates some ASD behavioral features and the neuroinflammatory status. Data provide the first pre-clinical indication for a therapeutic strategy against gut-immune activation in ASD subjects with peripheral increase of gut-derived (α4β7+) lymphocytes expressing IL-17A.

1. Introduction

The increased Autism Spectrum Disorder (ASD) risk related to maternal infections and/or inflammatory conditions or antibiotics use during pregnancy or early in life in children focuses on the possible interference of these conditions with the immune system and with the mucosal immune-response and microbiota-bacterial metabolites composition (Peralta-Marzal et al., 2021; Robinson-Agramonte et al., 2022; Vuong and Hsiao, 2017). This latter point is supported by the observations, within ASD populations, that: i) gastrointestinal disorders are frequently experienced and contribute to severity of behaviors seen in the disorder; ii) the gut microbiota shows a different composition compared to typically developing individuals (Settanni et al., 2021). Taken together, the above-reported information highlights the complex interplay between gastrointestinal tract and brain (the gut-brain axis), including the gut-environment interface represented by microbiota. In fact changes in microbiota composition (dysbiosis), primary or secondary to altered mucosal immune-response, influence brain activity

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and neuroinflammation as well as the perpetuation of a dysregulated mucosal immune response (Srikantha and Mohajeri, 2019).

A substantial body of evidence in animals and humans indicates the neuroimmune system as a key player in ASD pathogenesis (Hughes et al., 2023; Matta et al., 2019), as evidenced by activation of microglia and astrocytes and altered inflammatory cytokine levels in postmortem brain samples, cerebrospinal fluid (CSF) and serum plasma samples from individuals with ASD (Ashwood et al., 2011; Han et al., 2021; Vargas et al., 2005).

Moreover, a growing body of preclinical evidence is also suggesting a link between neurobehavioral symptoms and gut–brain axis dysfunction (Hosie et al., 2022; Hsiao et al., 2013). For instance, Li and colleagues found that offspring exposed to maternal immune activation (MIA) exhibited, beside the well characterized behavioral deficits, intestinal integrity disruption, microbial imbalances and gut inflammation (Kim et al., 2022; Li et al., 2021). Collectively these data support the importance to elucidate the contribution of gastrointestinal alteration in ASD.

In the present study, we attempt to clarify the contribution of mucosa immune response to ASD neuroinflammatory and behavioral profiles using the early-life immune activation (EIA) mouse model, a variant of the maternal immune-activation (MIA) model. EIA is a double-hit model including both prenatal (Poly I:C) and postnatal (LPS) immune challenges (Carlezon et al., 2019). In this model we evaluated the contribution of intestinal inflammation to the changes in behavior and neuroinflammation by administration of a monoclonal antibody directed against the integrin alpha4 beta7 (α4β7 mAb), responsible for leucocyte homing in the gut mucosa. Remarkably, the anti-α4β7 mAb has an effect limited to the gut compartment, causing a selective blockade of inflammatory monocytes and gut-experienced lymphocyte homing to the intestinal lamina propria.

2. Materials and methods

2.1. Animals and treatments

All animal procedures were performed in accordance with the European and National legislation (EU 63/2010, DL 26/2014) and protocols were approved by Institutional Animal Care and Use Committee (authorization n: 136/2021-PR). C57BL6/J [Segmented Filamentous Bacteria (SFB) positive] mice (Kim et al., 2017) were purchased from Charles River and housed under standard laboratory conditions (temperature 21° ± 1 °C, relative humidity 60 ± 10 %) and reversed 12-h dark–light schedule (lights off at 7:00 AM). As outlined in Fig. S1, ten days after their arrival, mice were mated, and females inspected for presence of a vaginal plug starting 12 h later [gestational day (GD) 0.5)] and twice a day in the following week. Pregnant females were left undisturbed until GD 12.5 when they were weighed and randomly assigned to intraperitoneal (ip) injections of either 20 mg/kg Poly(I:C) (0.01 ml/g) [potassium salt; P9582 (supplied as 10 % of the total weight of the salt) Sigma, St. Louis, MO] or saline. On postnatal day (PND) 9, pups were subcutaneously injected with either with 10 mg/kg LPS from Escherichia coli 0111:B4 (Catalog #L3024, Sigma-Aldrich) [prenatal Poly (I:C) offspring] or saline [prenatal saline offspring]. Pups then remained with the mother until PND 23–25, when they were weaned and mice from different mothers/cages were housed in same-sex/same treatment couples (no littermates). A total number of 17 litters were used (8 EIA and 9 saline). No significant difference in weight was observed between saline and EIA challenged animals during the second postnatal week.

Around the 6th postnatal week, male mice were ip injected each other day, with either anti-α4β7 blocking monoclonal Antibody (DATK-32, e-Bioscience US) or isotype control (rat IgG2a), 100 μg per mouse for seven injections (two weeks). Homing of α4β7, expressing leukocytes is mediated by the specific interaction between α4β7 and its ligand MAdCAM-1, which is expressed under steady state conditions by gut endothelial cells. Behavioral tests were carried out in two days after the fourth ip injection of anti- α4β7 mAb or its isotype during the dark phase of circadian cycle. Sequence of behavioral testing was: (1) open field, (2) three chamber social test, (3) marble burying.

To better describe sensorimotor competence, motor function and coordination were also tested by rotarod test. Rotarod training test was performed at 6 weeks of age before ip treatment as well as after ip treatment with anti-α4β7 mAb or its isotype.

2.2. Open field test

Around the 8th postnatal week, mice were tested in an open-field apparatus, a black plastic rectangular arena (40 × 40 × 40 cm) with grey floor. The session started placing the animal in one corner of the arena and lasted 40 min. Activity was recorded by a suspended video camera and analyzed using AnyMaze software (Stoelting Europe, Ireland). A central square zone (30 × 30 cm) was defined to evaluate number of visits and time spent into the central area (an anxiety-related response) (Vigli et al., 2020). Total distance moved, speed and number of entries into the central zone of the arena and immobility episodes were recorded across four 10-min as well as number of fecal boli at the end of test. The arena was cleaned with 50 % ethanol after each test.

2.3. Three-chamber social behavior test

The following day mice were tested in a 60 × 40 cm Plexiglas box divided into three chambers (with a doorways along each dividing walls to access to the two side chambers) (Tartaglione et al., 2022). The subject mouse was allowed to acclimate to the apparatus for 20 min before the sociability test, 10 min in the central chamber with the doors closed (session 1, S1), followed by 10 min in the entire empty arena with the doors open (S2), to freely explore the entire apparatus, including the two side compartments. The subject was then briefly confined to the center chamber while an inverted wire cup (10 cm height, 10 cm bottom diameter, bars spaced 0.8 cm apart) containing a plastic object (50 ml empty vial) was introduced into one of the side chambers. An unfamiliar, same-sex and strain mouse stranger enclosed in an identical wire cup was placed in the other side chamber. Doors were re-opened, and the subject was allowed to access to all three chambers for 10 min (S3). The subject was again confined to the center chamber and another unfamiliar same-sex and strain mouse was placed in the wire cage ‘previously containing the object, as a novel social stimulus; after both wire cups were re-positioned, doors were re-opened and the subject allowed to access to all three chambers and mouse behavior recorded for additional 10 min (S4). Side chamber location of the novel object and the stranger mouse were randomized across subjects. Measures taken included time spent in each chamber, time spent sniffing each cup, and number of entries. All testing chambers were cleaned with 50 % ethanol after each test. Animals used as “strangers” were same sex C57BL6/J aged 10–14 weeks old previously habituated to the apparatus and to the wire cup enclosure, 10 min per day for three days.Tests were video recorded and video files were subsequently analyzed with Observer 10XT (Noldus, NL); the 10 min sessions were divided in three intervals of 200 s duration and because of rapid habituation profiles, only the first interval was considered in line with previous data (Carlezon et al., 2019);frequency and duration of sniffing, cage rearing, rearing, wall rearing, grooming was scored by an experimenter blind to subject treatment.

2.4. Marble burying test

The marble burying assay, a test for repetitive/compulsive behavior, was also conducted 3 h after social behavior testing. Clean cages (36 × 20.5 × 18.5 cm) were filled 5 cm deep with sawdust bedding. The animals were first habituated in the cage only with bedding for 10 min. After habituation, the test mouse was gently removed from the cage. Then, 15 identical navy-blue marbles (1.2 cm diameter) were placed in a 3 × 5 rectangular matrix occupying 2/3 of the cage, and mouse was

allowed to explore the cage for a 10 min test period (Vigli et al., 2020). A marble was considered buried when the bedding covered more than 50 % of its surface area. video tracking analysis by Anymaze software was the same described above for the open field test.

2.5. Rotarod test

Motor function and coordination were tested using an accelerated rotarod device (Columbus Instruments, Columbus, OH, USA) according to the method described by Horvath et al. 2019 (Horvath et al., 2019). Mice were trained at constant 4 rpm speed for three consecutive trials to achieve the ability to maintain balance on the rod for at least 30 s. Acceleration phase testing (4–40 r.p.m. over 300 s) was performed on two subsequent days; four trials per day were carried out, with 30 min breaks between them, during which the mouse was put back into its home cage. Latency to fall from the accelerating rotarod was recorded.

2.6. Intestinal permeability

Twenty-four hours after behavioral testing and the last anti-α4β7 blocking mAb or isotype ip administration, a group of mice was administered with 60 mg/100 g body weight of FITC-conjugated dextran dissolved in purified water (4000 mw, Sigma-Aldrich) by gavage. Whole blood was obtained via eye bleed four hours after FITC-dextran administration. Blood samples were centrifuged at 3000 rpm to obtain sera; the latter were then analyzed by fluorometry using the Victor3™ (485/535 nm, gain 1480) plate reader (Perkin-Elmer).

At the end of behavioral and intestinal permeability testing, mice were sacrificed and CNS (hippocampus, prefrontal cortex, and cerebellum), colon and ileum regions, mesenteric lymph-nodes, and spleens were collected. All studies were carried out in accordance with the European and Italian legislation (2010/63/EU, DL 26/2014), project number N.136/2021.

2.7. Quantification of gene expression using RT-PCR

Male offspring were sacrificed around the 8^{th} postnatal week and tissue samples of hippocampus, prefrontal cortex, cerebellum, ileum and colon were collected and stored at –80 °C. Total RNA was extracted using Trizol reagent (Invitrogen, Thermo Fisher Scientific) for hippocampus, prefrontal cortex and cerebellum and RNA mini Kit Plus (Qiagen) for ileum and colon. The quality and concentration were measured at Nanodrop. cDNA was reverse transcribed from 1 μg of RNA using the High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems™ - Thermo Fisher Scientific). The gene amplification was performed in duplicate at Tm-60 °C using TaqMan master mix and TaqMan™Gene Expression Assays (both from Applied Biosystems). As housekeeping gene, we utilized hypoxanthine guanine phosphoribosyl transferase (HPRT). The relative expression level of each mRNA was calculated using the $2^{-\Delta\Delta Ct}$ method, normalized to HPRT and relative to saline isotype mice.

2.8. Mesenteric lymph-nodes (MLN) lymphocytes isolation

Lymphocytes of mesenteric lymph-nodes were isolated as described (Qiu and Sheridan, 2018). In brief, after cecum localization, MLN chain was exposed and removed. MLNs were dissected after rolling the chain on a paper towel to remove the fat. Lymphocytes were isolated by gently dissociation through a 100-μm cell strainer using the plunger of a 3-mL syringe. After washing, viable cells were assessed by trypan blue exclusion.

2.9. Spleen cell isolation

Splenocytes were isolated from freshly spleen specimens by gentle mashing with a syringe plunger and filtered through a 100-μm mesh (BD biosciences, USA). After centrifugation for 7 min at 1500 rpm in PBS, the red blood cells were lysed using ammonium–chloride–potassium (ACK) lysing buffer. After washing, viable cells were assessed by trypan blue exclusion.

2.10. Immunofluorescence

Viable cells ($1x10^6$/ml) isolated from MLN and spleen were incubated in separated wells for 4 h at 37 °C in a humid 5 % CO2 atmosphere in complete medium (RPMI 1640 plus 10 mM HEPES buffer, 2 mM L-glutamine, 10 % heat-inactivated FCS (Hyclone), and antibiotics) left unstimulated or stimulated for 4 h at 37 °C in complete medium with Phorbol-12-myristateacetate (PMA) at 50 ng/ml, ionomycin (Sigma-Aldrich) at 1 μg/ml and Golgi Stop solution at 0.66 μl/ml (BD Pharmingen). After incubation, cells were harvested, washed and incubated for 30 min with Fixable Viability Dyes eFluor™ 780 (eBioscience™, ThermoFisher). Next, cells were washed and FcγIII/II receptor (BD bioscience) was added to block non-specific Fc receptor binding of antibodies. In immunofluorescence panel setting experiments, we observed, as previously reported (Kaldjian et al., 1988), a down-regulation of CD4 expression after PMA-ionomicyn stimulation, while CD8 expression was not affected (Fig. S2). Therefore, CD8 staining was preferred and $CD4^+$ cells were defined as $CD3^+$ $CD8^-$ ($CD4^+$) cells. Cells were stained with anti-mouse CD3 – PE-CF594 (BD bioscience), CD8a – Alexa Fluor 700 and anti-α4β7 - BV650 (ThermoFisher) or corresponding isotypes. After incubation, cells were washed, fixed, permeabilized with fixation/permeabilization buffer (eBioscience, ThermoFisher) for 40 min and stained with anti-mouse IFN-γ – BV786 (ThermoFisher) and IL17A – BUV395 (BD bioscience) or corresponding isotypes. The percentage of viable fluorescent cells was quantified using a CytoFLEX LX (Beckman Coulter).

2.11. Statistical analyses

Data from behavioral tests and relative mRNA expression data of males were analyzed by two way ANOVA to take into account main effect of condition (Saline vs EIA), main effect of treatment (isotype vs anti-α4β7 ab) and their interaction followed by Tukey's Post-hoc tests (see Tables S1–S3).

Behavioral data (OPF, Three-Chamber) also containing repeated measurements within the same subject were analyzed by mixed-model ANOVA (see Table S1) with condition and treatment (two-way) as between-subject factor and repeated blocks (when present) as within-subject factors. Post-hoc comparisons were performed using Tukey's HSD test.

Rotarod data before ip treatment were analyzed by mixed-model ANOVA [condition (EIA-saline) × repeated blocks (trials)] followed by Tukey's Post-hoc tests. As rotarod data collected after anti-α4β7 and isotype treatment did not matched the assumptions for normality we applied non-parametric Mann-Whitney for each day trial (9–16) followed by Monte Carlo permutation test due to the small sample size (Hayes, 1996).

Female behavioral data were analyzed with mixed-model ANOVA [condition (saline or EIA) × repeated measurements and their interaction]. Relative mRNA expression data of females were analyzed by Student's *t* test (two tailed) (saline vs EIA condition).

Immunofluorescence and intestinal permeability data were compared using the context independent Student's *t* test (two tailed) (Sinclair, 1988).

Analyses were performed with GraphPad Prism 9.4.

3. Results

3.1. EIA male mice show a significant increase of IL-1β and IL-17A mRNA content in the colon and increased intestinal permeability. Blockade of gut α4β7 mediated leukocytes homing is associated with colonic gut immune-response and intestinal permeability comparable to controls.

We started our observations analyzing the effects in mice of EIA on gut immune response. Since in the EIA model sex differences in long-term effects of the immune-activation have been reported (Carlezon et al., 2019), our initial observations included both male and female mice. However, in female mice we did not observe significant differences in gut immune response when compared with controls, although, as previously reported (Carlezon et al., 2019), we observed decreased locomotor activity and neuroinflammation (in all likelihood gut-independent) (Fig. S3A–D, Tables S1–S3). Therefore, in the following studies, we focused on male mice.

We observed a significant increase of IL-1β and IL-17A mRNA colonic tissue content in EIA-isotype male mice when compared with controls (saline-isotype) (Fig. 1A and Table S1). This increase was restricted to the colon and was not observable in the ileum (Fig. 1B). Administration of anti-α4β7 mAb was associated with a significant reduction of IL-1β and IL-17A in EIA treated mice that showed values comparable to controls (saline-isotype and saline-anti-α4β7, Fig. 1A). Notably, administration of anti-α4β7 mAb is associated with a broad inhibition of monocyte-derived inflammatory cytokine (e.g. IL-6 and TNF-α). Thus, administration of α4β7 blocking Ab was able to reduce mRNA colonic content of inflammatory cytokines in EIA mice.

Given the close two-way relationship between intestinal inflammation and intestinal permeability, we measured intestinal permeability in male mice. As shown in Fig. 2, we observed an increase of intestinal permeability in EIA-isotype mice when compared to saline-isotype controls (2294 ± 288 vs 1605 ± 163, mean ± SE, $p < 0.05$) while EIA mice treated with anti-α4β7 mAb show values comparable to controls.

3.2. EIA male mice show an impaired response to social novelty in the three-chamber test. Treatment with anti-α4β7 mAb restored the social novelty response

A selective effect of anti-α4β7 treatment, favoring social responsiveness in EIA, but not in saline treated mice, was evident when subjects can visit either the compartment with an unfamiliar same sex partner (social stimulus) or the compartment with an inanimate object (object) [Fig. 3A left panel, $p < 0.05$ after post hoc comparisons performed on the interaction condition × i.p. treatment $F(1,41) = 5{,}00$, $p = 0.03$].

In the following session, when the tested mouse can choose between a familiar and a novel social stimulus, a selective impairment in social novelty response was evident in EIA-isotype mice. Such deficit is fully re-established by anti-α4β7 treatment, that brings back exploration of novel partner to control levels [see Fig. 3A right panel, $p < 0.05$ after post hoc comparisons performed on the interaction condition × ip treatment $F(1,41) = 11{,}22$, $p = 0.0017$].

In the open field test, considering the entire 40 min of the test, EIA and saline mice displayed comparable levels of locomotor activity (distance moved, mean and max velocity, number and duration of immobility episodes and time spent in central area of the arena) and comparable number of fecal boli were found at the end of testing (data not shown); as expected, total distance traveled significantly decreased in the throughout the four 10 min intervals [main effect of repeated trials: $F(3,123) = 148.09$, $p < 0.001$] and no treatment × repeated 10 min-block interactions were evident. Only by the end of the test, focusing on the last 10 min interval, it was evident that EIA mice were displaying a disinhibited exploratory profile, with increased time in the central zone in comparison with saline mice [main effect of EIA condition $F(1, 41) = 5.00$, $p < 0.05$]; moreover, distance moved in the central area in EIA-isotype mice was significantly longer than both saline-isotype mice and EIA anti-α4β7 [ps < 0.05 after post hoc comparisons $F(3, 41) = 3.71$, $p = 0.06$], and number of entries in the central area were greater in EIA-isotype mice than in EIA anti-α4β7 [$p < 0.05$ after post hoc comparisons $F(3, 41) = 4.46$, $p = 0.04$] (Fig. 3B and Table S2). A subsequent video analysis of the behavioral responses displayed animals during the last 10 min interval of the open field test did not reveal any difference in frequency and duration of grooming, wall rearing or jumping (data not shown), suggesting that such disinhibited exploratory profile is not associated with the onset of stereotypic responses. This is also confirmed by the absence of any main effect of condition or ip treatment (or their interaction) in the marble burying test.

3.3. EIA male mice show impairment of motor learning, ameliorated by anti-α4β7 mAb administration

At six weeks of age EIA male mice showed an impairment in motor learning, measured using a rotarod task, compared with saline mice, with significant differences throughout trials between groups (Fig. 4A; treatment× trial interaction [$F(7,217) = 2.499$, $p = 0.017$]).

After anti-α4β7 mAb administration, EIA mice showed an improvement in motor skill consolidation (Fig. 4B, trial 9–16); the second day of rotarod test, EIA anti-α4β7 mice increased time spent on the rotating rod compared to EIA isotype mice with a significant rescue in motor performance in the last trial (trial 16, $p < 0.05$ Monte Carlo permutation results for Mann-Whitney test using 10,000 permutations).

No differences were found in saline animals (Fig. 4C, trial 9–16) supporting the absence of anti-α4β7 mAb or its isotype toxicity on mouse motor functions.

3.4. EIA differentially affects the neuroinflammatory profile in the hippocampus, prefrontal cortex and cerebellum of male offspring: effects of treatment with anti-α4β7 mAb

To get insights into the CNS alterations associated with behavioral changes in EIA male mice and the effects of gut immune-activation tempering, we examined the expression of a set of genes involved in homeostatic and immune functions of microglia and markers of neuroinflammation, in the hippocampus, prefrontal cortex and cerebellum of adult offspring. Specifically, we analyzed the mRNAs expression of the pro-inflammatory cytokines TNF-α, IL-6 and IL-1β, the anti-inflammatory cytokine TGF-β, the inflammatory/oxidative stress-related enzyme iNOS, the microglia/macrophage phagolysosomal marker CD68, and the neurotrophin Brain-Derived Neurotrophic Factor (BDNF). In the hippocampus (Fig. 5A), we found elevated levels of mRNAs for IL-6 and IL-1β and TNF-α in EIA male mice when compared with saline controls, while the expression of TGF-β, CD68 and total BDNF mRNAs were significantly reduced in EIA-isotype vs. saline-isotype. Administration of anti α4β7 mAb significantly rescued the expression of TGF-β, CD68 and BDNF mRNAs in the hippocampus of EIA treated mice with values comparable to controls (saline-isotype and saline-α4β7), while it did not affect IL-6, IL-1β and TNF-α changes.

In the prefrontal cortex of EIA mice (Fig. 5B), similarly to the hippocampal profile, we found elevated levels of IL-6 and IL-1β, while the levels of the expression of TNF-α and TGF-β were not modified by EIA conditions. CD68 mRNA was increased in EIA mice when compared to saline mice. Similarly to what found in the hippocampus, the levels of the total BDNF mRNA were significantly reduced in EIA-isotype mice *vs* saline isotype. Administration of anti-α4β7 mAb in EIA mice was associated with an increase of BDNF mRNA expression that became comparable to the one observed in sal-isotype mice and sal-α4β7. Anti-α4β7 mAb administration had no effect on IL-6 and IL-1β and CD68 expression. Both in the hippocampus and in prefrontal cortex iNOS mRNA did not change in any of the examined conditions (data not shown).

In the cerebellum of EIA mice (Fig. 5C), we found an increase of the expression of TNF-α and a significant reduction of TGF-β and BDNF mRNA in EIA mice. The other mRNAs analyzed (IL-6, IL-1β, CD68, and

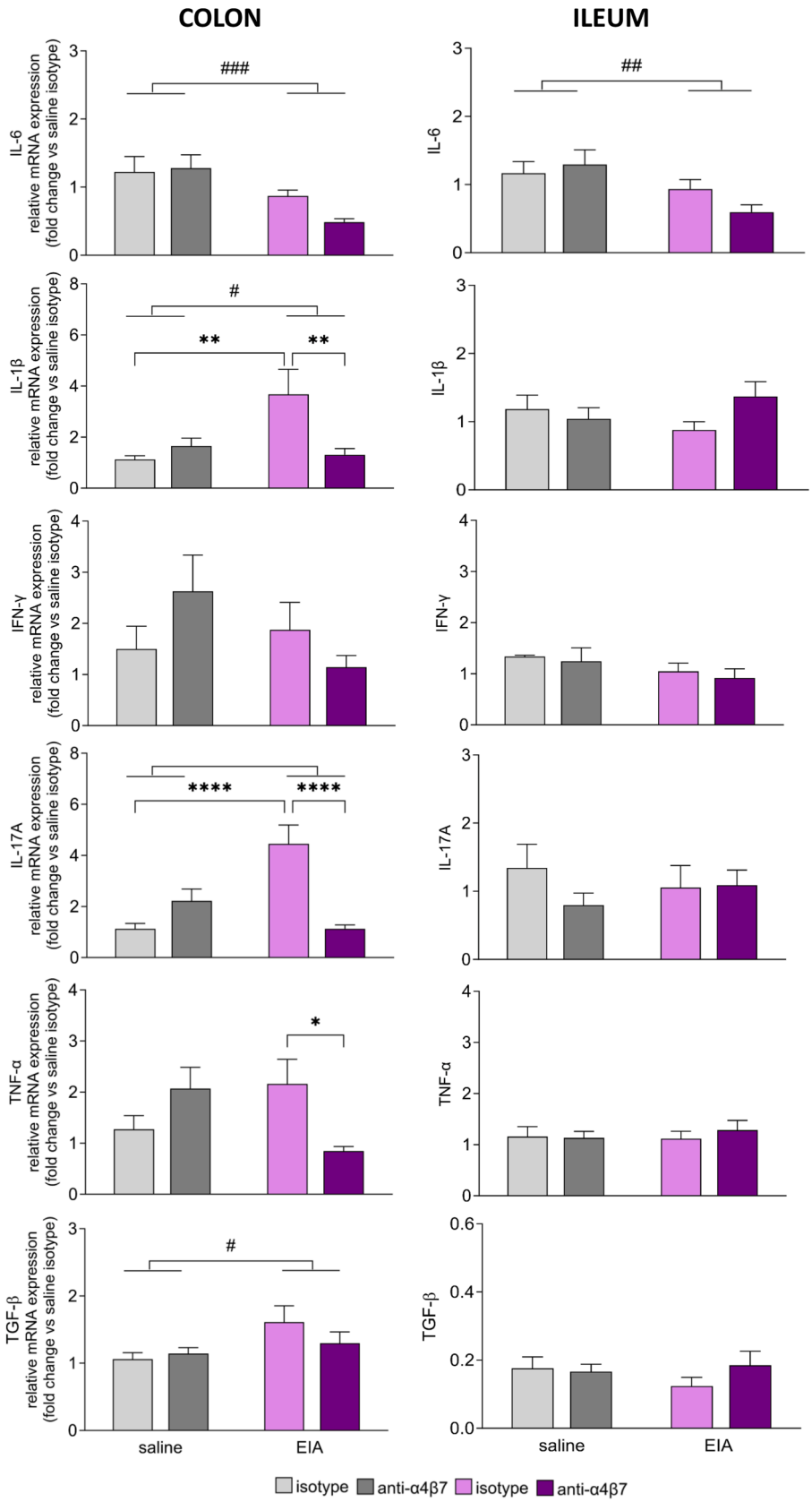

Fig. 1. Effect of anti-α4β7 monoclonal antibody or isotype antibody administration on gene expression of cytokine in colon (left panel) and ileum (right panel) tissue. mRNA tissue content quantified by real-time PCR. The relative expression level of each mRNA was calculated using the $2^{-\Delta\Delta Ct}$ method, normalized to HPRT and relative to saline-isotype mice Data were analyzed by two way ANOVA (see Table S1) and represented as mean + SE. Data are from 8 to 12 mice per group. Main effect of condition: # $p < 0.05$; Tukey's post hoc comparison: *$p < 0.05$, ** $p < 0.005$, *** $p < 0.0005$; **** $p < 0.0001$.

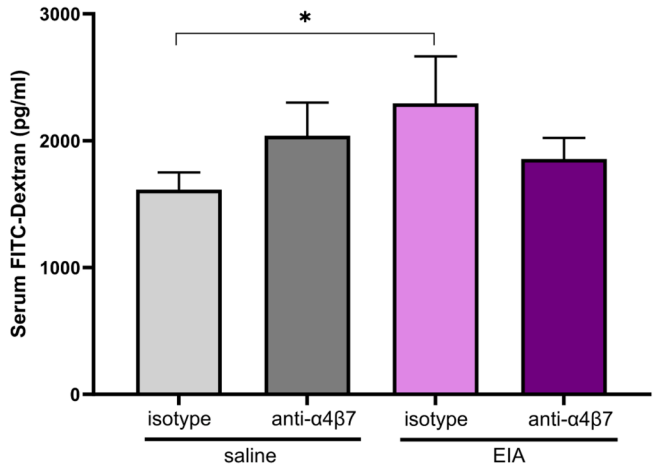

Fig. 2. Intestinal permeability. Mice were administered with a dose of 60 mg/100 g body weight of FITC-conjugated dextran dissolved in purified water by gavage. Whole blood was obtained four hours after FITC dextran administration. Columns represent mean + SE. * $p < 0.05$ from Student's t (two tailed) test. Saline isotype n = 8; saline anti-α4β7 mAb n = 7; EIA isotype n = 5; EIA anti-α4β7 mAb n = 7.

iNOS) were not altered by EIA condition. Administration of anti-α4β7 mAb did not modulate the expression of any of the genes analyzed.

3.5. EIA mice show increased percentage of CD4$^+$ IL-17A$^+$ and CD4$^+$IFN-γ$^+$ lymphocytes in MLN and spleens that was reduced by treatment with anti-α4β7 mAb

In the attempt to explore the influence of the gut microbiota composition on inductive site of gut immune-response, we analyzed isolated mesenteric lymph-nodes (MLN) lymphocytes in EIA mice and controls by immunofluorescence. We observed in EIA mice a significant increased % of CD4$^+$ (defined as CD3$^+$CD8$^-$ (CD4$^+$) cells see methods *1.2.10*) IL-17A$^+$ and CD4$^+$α4β7$^+$IL-17A$^+$ lymphocytes both in unstimulated and "in vitro" stimulated conditions when compared with mice treated with saline-isotype. A significant increased percentage of CD4$^+$IFN-γ$^+$ and CD4$^+$α4β7$^+$IFN-γ$^+$ was also observed in EIA mice when compared to control mice, but only in "in vitro" activated lymphocytes. Notably, EIA mice treated with anti-α4β7 mAb showed values comparable to controls (see Fig. 6A and Fig. S3 B-C).

We also analyzed isolated splenic lymphocytes as expression of peripheral immune-activation in the EIA model. We found an increased percentage of unstimulated and "in vitro" stimulated CD4$^+$α4β7$^+$IL-17A$^+$ lymphocytes. We also found an increased % of "in vitro" stimulated CD4$^+$α4β7$^+$IFN-γ$^+$ lymphocytes when compared to controls. In mice treated with the anti-α4β7mAb, % of IL-17A$^+$ and IFN-γ$^+$ CD4$^+$α4β7$^+$ lymphocytes were similar to controls (see Fig. 6B and Fig. S3 D-E).

4. Discussion

In the present study, we evaluated the contribution of gut immune-response to the ASD phenotype and associated CNS key homeostatic/inflammatory genes in the ASD mouse model of early-life immune activation (EIA). We found that blockade of innate cells and gut-experienced lymphocytes traffic by means of anti-α4β7 monoclonal antibody administration was able (as expected) to inhibit the gut inflammation present in the EIA treated animals, normalize intestinal permeability and that these features were associated with rescue of social responsiveness and expression of protective genes in hippocampus and prefrontal cortex.

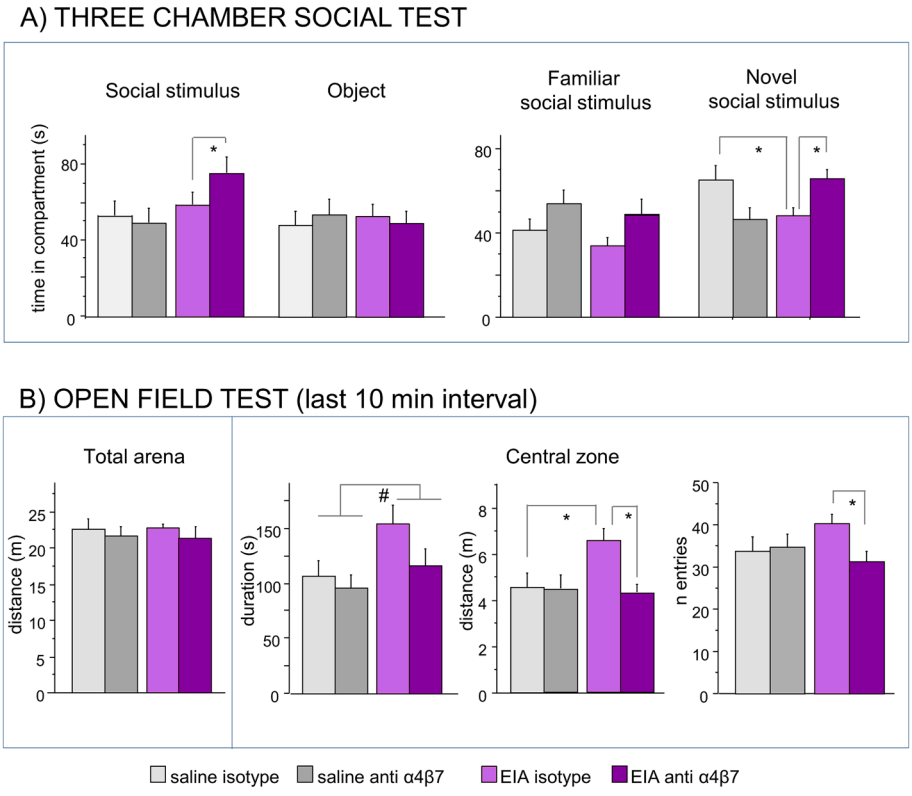

Fig. 3. Behavioral profile. A) Three-chamber test. Left panel: time spent in the compartment containing the social stimulus or the inanimate object. Right panel: time spent in the compartment containing the familiar social stimulus or the novel social stimulus. B) Open field test (data reported are from the last 10 min interval). Data are mean + SE; main effect of condition (saline vs EIA): # $p < 0.05$; * $p < 0.05$ after post hoc comparisons on ANOVA (see Table S1). Saline Isotype n = 10; Saline anti-α4β7 mAb n = 12; EIA Isotype n = 10; EIA anti-α4β7 mAb n = 13.

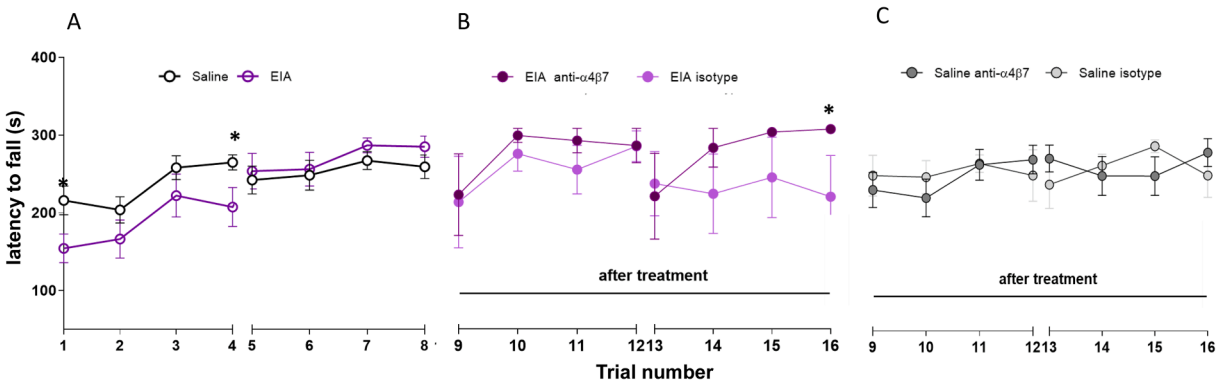

Fig. 4. Motor coordination. Motor function and coordination were tested in offspring mice of 6 weeks of age before (panel A, trial 1–8) and after (trial 9–16) anti-α4β7 mAb or its isotype administration (panel B and C). Testing phase was performed on two subsequent days (four trials/day). Data are shown, for each trial, as mean latency to fall ± SE. Panel A * $p < 0.05$, after post hoc comparisons on ANOVA; Panel B * $p < 0.05$ after Monte Carlo permutations for Mann-Whitney test using 10,000 permutations. Saline = 22; EIA n = 11; Saline Isotype n = 6; Saline anti-α4β7 mAb n = 10; EIA Isotype n = 4; EIA anti-α4β7 mAb n = 5.

In the EIA model, we observed a gut inflammation characterized, in the colon, by an increased IL-1β and IL-17A mRNA tissue content and by an increased percentage of stimulated $CD4^+$ cells expressing IFN-γ and $CD4^+$ cells expressing IL-17A in the MLN and spleen. Notably, in the spleen, the increase was restricted to the $CD4^+\alpha4\beta7^+$ subset as expression of gut-primed lymphocytes. Percentage of $CD4^+IL\text{-}17A^+$ and $CD4^+IFN\gamma^+$ lymphocytes was increased in the inductive MLN site of mucosal immune-response suggesting a role of the microbiota in its genesis. This increase is mirrored in the periphery (spleen) and observed at effector site (colon) as increased mRNA tissue content. However, in the colon, we observed an increase of IL-17A mRNA but not of IFN-γ mRNA tissue content, possibly as result of migration in the colon of "in vivo" expanded activated $CD4^+IL\text{-}17A^+$ lymphocytes. In fact, both in the MLN and in the spleen, we observed an increase of unstimulated $CD4^+\alpha4\beta7^+IL\text{-}17A^+$ lymphocytes revealing "in vivo" expanded cell populations that further increase after "in vitro" activation. As for $CD4^+\alpha4\beta7^+IFN\text{-}\gamma^+$ lymphocytes, instead, we observed an increase only in the "in vitro" activated cells, suggesting that, in the colon, these cells are not actively producing mRNA. The presence of gut inflammation was not specifically investigated in the EIA model, which represents a variant of the maternal immune-activation (MIA) model. However, similarly to our observations, a proinflammatory T-helper-cell phenotype was described in the MIA model characterized by an early onset and persistent increased release of IL-17A from $CD4^+$ T cells from spleens and MLN (Hsiao et al., 2012).

As reported in the MIA model (Hsiao et al., 2013), we observed also in the EIA model a significant increase of intestinal permeability which may be related to the increased IL-1β colonic tissue content. Indeed, IL-1β (increased in the colon of EIA mice and comparable to controls in the anti-α4β7 treated mice in the present study) is able to increase intestinal permeability in part by the activation of the canonical NF-kB pathway, Mitogen-Activated Protein Kinases (MLCK) gene activation and post-transcriptional degradation of occludin mRNA (Kaminsky et al., 2021). The observation that therapeutic targeting of the IL-1β-induced increase in intestinal permeability is effective in ameliorating colitis in the murine DSS model of colitis further reinforces the hypothesis (Rawat et al., 2020).

In our studies, administration of the anti-α4β7 monoclonal antibody was able to normalize the observed increase of IL-17A and IL-1β colonic mRNA tissue content as well as to reduce the content of innate cells-derived cytokine IL-6 and TNF-α. Indeed, in addition to the generally accepted ability of anti-α4β7 monoclonal antibody to reduce gastrointestinal inflammation by decreasing trafficking of gut-primed circulatory T cells into the intestinal mucosa (Lindebo Holm et al., 2012; Wang et al., 2010), a large body of evidence suggests that the effects of α4β7 targeting may extend beyond the T cell compartment. It has been demonstrated that protective effects on colitis associated with genetic deletion of β7 result from T cell-independent protective effects due to interference with colonic recruitment of proinflammatory monocytes (Schippers et al., 2016). Furthermore, it has been described a critical role of α4β7 in the regulation of dendritic progenitor cell trafficking to the intestine (Clahsen et al., 2015; Zeng et al., 2013) but not for the migration of activated DCs from the small intestine to MLN (Clahsen et al., 2015).

The behavioral profile in EIA male mice in our study presents two main alterations. The first one is an impairment in social novelty response in the Three-chamber test, in line with previous data (Carlezon et al., 2019); on this alteration, which is targeting a core feature of rodent social repertoire (Netser et al., 2020), treatment with anti-α4β7 monoclonal antibody exert a beneficial effect, and social novelty values in this group display levels comparable to saline controls. The second one (undoubtedly an unusual behavioral feature in mouse models for the study of ASD) consists of a disinhibitory profile characterized by a greater interest for the central zone of the arena (while peripheral area, near the wall is usually considered safer), where EIA mice stay longer and travel longer distances. This behavior is detectable only in the last 10 min interval of a 40 min open field test, after the expected decrease in locomotor activity (habituation) has occurred in all groups. Treatment with anti-α4β7 monoclonal antibody countered such profile and led to values of number of entries and distance moved in the central zone comparable to controls. These data cannot be compared with previous observations made in EIA male mice in a 20 min open field test (Carlezon et al., 2019). However, it may be considered as a behavioral expression of immune dysregulation (particularly of TGF-β) observed in the present study as well as in human ASD subjects (Ashwood et al., 2008).

Our data reveals that maternal and early postnatal immune activation (double hit) also induces an impairment of motor learning skills in male offspring tested with a rotarod device. Motor control and learning impairments are common complications in individuals with ASD (Hilton et al., 2012). Children with autism exhibit several motor deficits including poor coordination and delayed learning of complex motor skills (Gidley Larson et al., 2008) and decreased ability in rotarod test were already reported in ASD mouse models (Mapelli et al., 2022; Xiao et al., 2020). Performance on rotarod is not only the result of enhanced general locomotor activity: the length of time that mice stay on rotating rod is a measure of their balance, coordination and motor-planning, a range of behaviors and skill learning that require well-integrated processes, much more complex than those analyzed in spontaneous motor activity (Buitrago et al., 2004). In our experimental condition the anti-α4β7 mAb treatment was able to enhance motor learning in EIA mice, an effect that could be related to modulation of cortical BDNF expression induced by the antibody administration; indeed, cortical BDNF has been

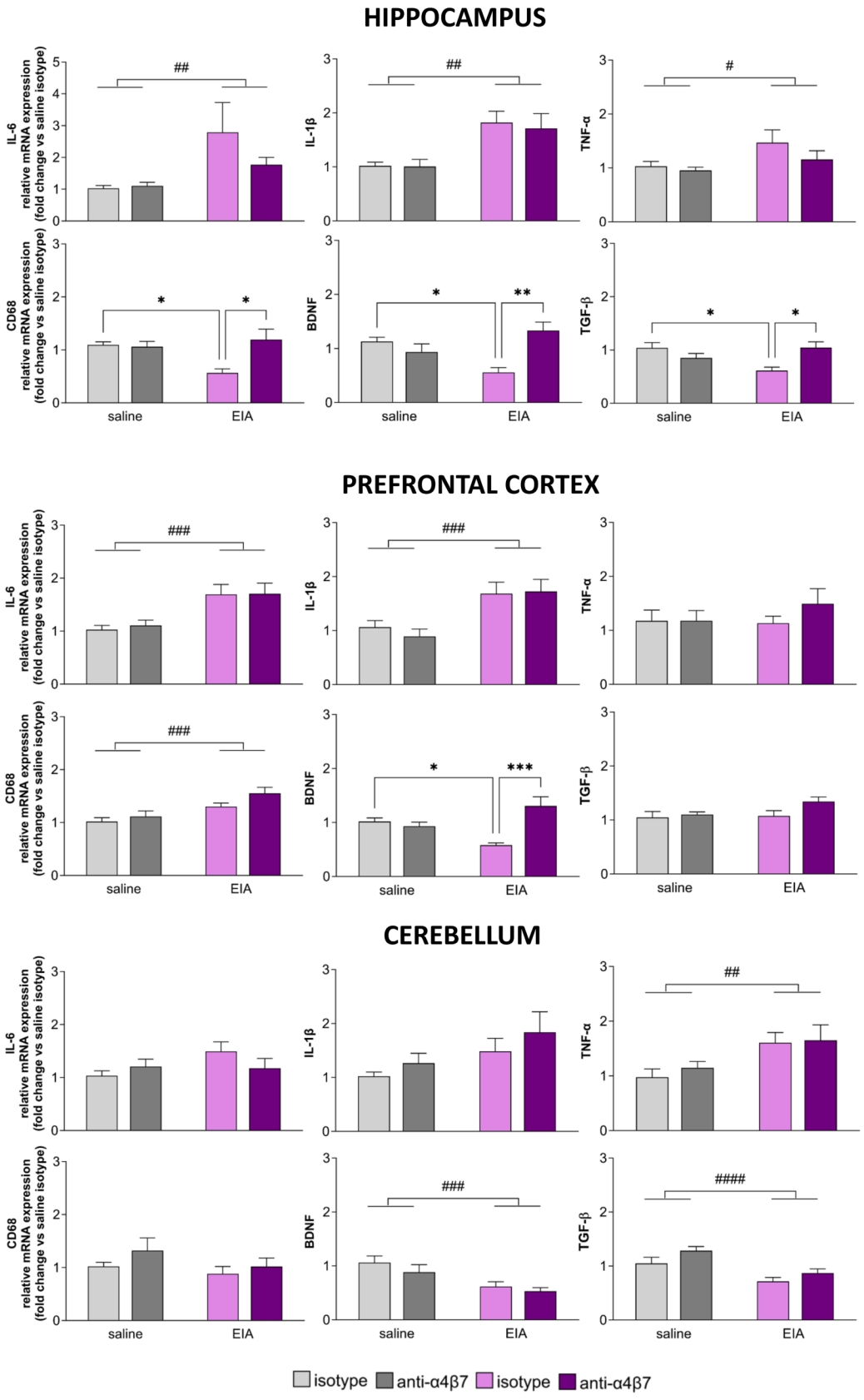

Fig. 5. Effect of anti-α4β7 monoclonal antibody or isotype antibody administration on gene expression in the Hippocampus (A), Prefrontal cortex (B) and Cerebellum (C) mRNA tissue content quantified by real-time PCR. The relative expression level of each mRNA was calculated using the $2^{-\Delta\Delta Ct}$ method, normalized to HPRT and relative to saline-isotype mice. Data are from 10 to 12 mice per group, expressed as mean + SE. Main effect of condition: # $p < 0.05$, ## $p < 0.005$, ### $p < 0.0005$, #### $p < 0.0001$; Tukey's post hoc comparison: *$p < 0.05$, ** $p < 0.005$, *** $p < 0.0005$.

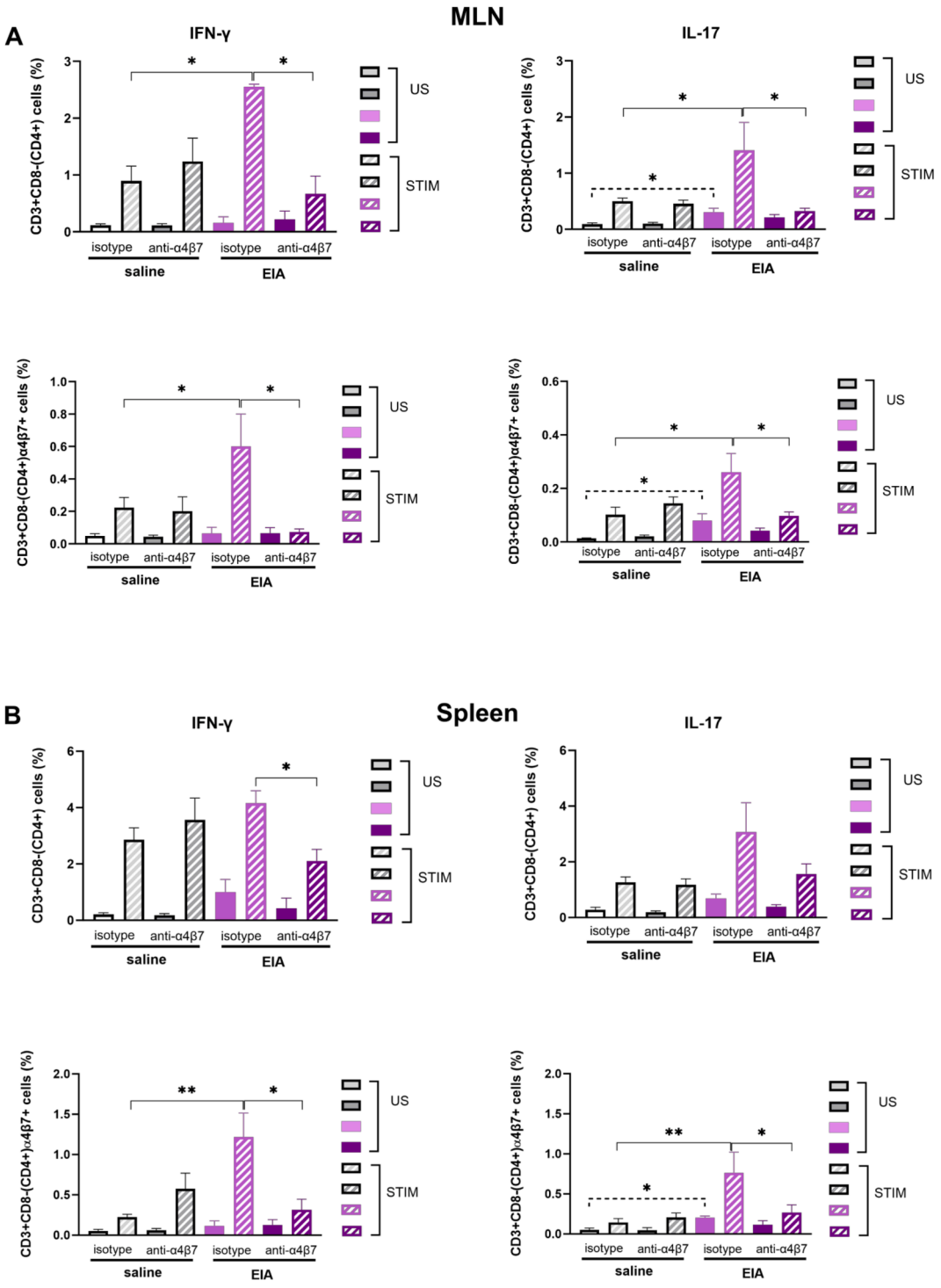

Fig. 6. Effect of anti-α4β7 monoclonal antibody or isotype antibody administration on % of $CD3^{+}CD8^{-}(CD4^{+})$ mesenteric lymph-node (MLN) (A) and spleen (B) isolated lymphocytes. Isolated lymphocytes were either left unstimulated or stimulated with PMA and ionomycin in the presence of Golgi stop for 4 h at 37 °C in complete medium. Cells were then harvested, stained and analyzed by immuno-fluorescence. Columns represent mean + SE. MLN and Spleen: Saline isotype n = 6; saline anti-α4β7 mAb n = 8; EIA isotype n = 3; EIA anti-α4β7 mAb n = 4. * $p < 0.05$; ** $p < 0.005$ by Student's (two tailed) *t* test.

reported essential for motor learning (Andreska et al., 2020). Furthermore, as decreased rotarod performance can be induced by LPS-activated microglia (Patro et al., 2010), beneficial effects of anti-α4β7 mAb could be also related to its ability to modulate microglial activation.

As for analysis of CNS gene expression, results highlight long-lasting effects of the double-hit EIA regimen on the expression of key homeostatic/inflammatory genes, including the neurotrophin BDNF, the macrophage/microglial marker CD68, and the cytokines IL-6, IL-1β, TNF-α and TGF-β in the brain of adult male mice. Remarkably, we show that the gut-selective integrin-targeted therapy with the anti-α4β7 monoclonal antibody was able to rescue altered expression of BDNF mRNA in the hippocampus and cortex, and of TGF-β and CD68 in the hippocampus.

Downregulation of total BDNF mRNA in EIA-isotype mice in all regions examined (i.e. hippocampus, prefrontal cortex and cerebellum) is

in line with previous data in which, a similar downregulation of brain BDNF level was associated with behavioral disturbances relevant to the pathology (De Simone et al., 2020; Dutra et al., 2023; Tartaglione et al., 2022). Alterations of BDNF homeostasis and downstream signaling are considered contributing factors to ASD pathogenesis (Barbosa et al., 2020; Camuso et al., 2022; Robinson-Agramonte et al., 2022); whereas increased BDNF levels in hippocampus and prefrontal cortex have been documented following physical and social enrichment in mice (Cirulli et al., 2010); it is therefore conceivable that the anti-α4β7 mAb-induced increase of BDNF could underlie both the increased social responsiveness in EIA condition as well as the rescue of the social novelty impairment.

In the hippocampus of EIA mice, the reduced levels of CD68 – a marker usually used to assess the reactivity of microglia - could be indicative of a persistent reduction of microglia-mediated synapse elimination, which in turn could contribute to the behavioral alterations detected in EIA-isotype mice. Reduced hippocampal microglial phagocytic activity has been reported in male mice prenatally exposed to Poly I:C (Hui et al., 2018; Mattei et al., 2017). Importantly, the anti-α4β7 mAb treatment was able to fully restore hippocampal CD68 expression, favoring the hypothesis of a direct contribution of gut-derived signals on brain function.

Consistently with several preclinical studies, we also found brain region-specific regulations of other inflammatory genes. A prominent induction of IL-6 and IL-1β was observed in both hippocampus and cortex, while in hippocampus and cerebellum we observed an increase of TNF-α associated with a downregulation of TGF-β. Since main source of these inflammatory mediators is microglia, changes observed in EIA mice could actually reflect region-specific alterations in microglial activation profile.

A dysregulation of inflammatory cytokine levels such as IL-1β, IL-6, TNF-α and relative signaling pathways have been found in ASD children as well as in MIA offspring and associated with pronounced behavioral impairment (Carlezon et al., 2019; Erbescu et al., 2022; Garay et al., 2013; Mattei et al., 2017). As for TGF-β, a growing body of evidence indicates its implication in regulating cellular and behavioral plasticity underlying neuropsychiatric disorders, and decreased serum levels of TGF-β were reported in patients with autism (Mitra et al., 2022; Okada et al., 2007). mAb treatment was able to restore only the hippocampal levels of TGF-β in EIA mice, without affecting the regulation of the other cytokines, at variance with what observed in the colon.

Moving the attention on how the abolition of the intestinal inflammation can influence some aspects of the behavior and modulate protective genes in the CNS, it is necessary to underline that, in our study, this effect is obtained with the increase of protective factors acting on CNS rather than reducing observed inflammation (differently from local effects of α4β7 mAb inhibiting gastrointestinal inflammation). Useful for the interpretation of these results is the recent observation that IL-17A and IL-17F are modulators of intestinal homeostasis that indirectly alter CNS through the intestinal microbiome (Regen et al., 2021). Thus, the behavioral and CNS gene expression effects triggered by treatment with anti-α4β7 monoclonal antibody might be due to local modulation of microbiota and its metabolites acting on CNS and not be the result of a generalized anti-inflammatory effect. In fact, gut microbiota composition influences gut local immune-cell populations and their associated mediators and they in turn influence it. Dysbiosis and a concomitant increase of colon expression of cytokines and inflammatory markers has been described in MIA model, although it is not known whether the dysbiosis occurs as part of the immune dysfunction itself or is a result of it (Hsiao et al., 2013). Given the above considerations, it is tempting to speculate that the intervention on intestinal inflammation was able to influence the microbiota and its metabolites. In support of this hypothesis, evidence suggests that BDNF is influenced by dysbiosis and microbiota metabolites (Ahmed et al., 2022; Morris et al., 2017) and that in turn BDNF is able to influence behavior and, in particular, social interactions (Cirulli et al., 2010; Scattoni et al., 2013). Further studies are necessary to confirm this hypothesis.

The results of the present study point to clear beneficial behavioral effects in a mouse model for the study of ASD of a treatment targeting gut inflammation. One limitation of this study is related to the occurrence of intestinal inflammation in male mice only. To our knowledge, this is the first study to investigate this issue. However, in EIA female mice we observed decreased general locomotor activity (differently from EIA males) and neuroinflammation (in all likelihood gut-independent) as previously reported (Carlezon et al., 2019). Despite this limitation, we believe our findings may be of interest for development of therapeutic strategy in human ASD, especially in subjects with gastrointestinal symptoms. In these subset of patients that show dysbiosis and increased neuroactive microbiota-derived metabolites (Stewart Campbell et al., 2022) therapeutic intervention on microbiota and/or microbiota-derived metabolites (Stewart Campbell et al., 2022; Tu and Zhao, 2021) have been put in place with encouraging results.

5. Conclusion and future directions

Results of the present study suggest that a mucosal anti-inflammatory treatment might complement the above-reported therapeutic interventions, particularly in a subset of ASD subjects showing peripheral blood increased percentage of $CD4^{+}\alpha4\beta7^{+}IL\text{-}17A^{+}$ lymphocytes and indeed, an increased percentage of $CD4^{+}\beta7^{high}$ cells in “in vitro” stimulated peripheral blood mononuclear cells has been reported in ASD children with gastrointestinal symptoms (Rose et al., 2020).

Of note, an anti-α4β7 humanized monoclonal antibody approved to treat Inflammatory Bowel Disease, is already available and considered safe for pediatric use (Ibrahimova et al., 2021), thus facilitating translational purposes.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

Acknowledgements

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.bbi.2023.09.024.

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