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Research Article

Novel antibodies that selectively block mouse IL-12 enable the re-evaluation of the role of IL-12 in immune protection and pathology

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The dimeric cytokine IL-12 is important in the control of various infections but also contributes to the pathology of certain diseases making it a potential target for therapy. However, its specific inhibition with antibodies is complicated by the fact that its two subunits are present in other cytokines: p40 in IL-23 and p35 in IL-35. This has led to erroneous conclusions like the alleged implication of IL-12 in experimental autoimmune encephalomyelitis (EAE). Here, we report the development of a mouse anti-mouse IL-12 vaccine and the production of monoclonal antibodies (mAbs) that do not react with p40 or p35 (in IL-35) but specifically recognize and functionally inhibit the IL-12 heterodimer. Using one of these mAbs, MM12A1.6, that strongly inhibited IFN-γ production and LPS-induced septic shock after viral infection, we demonstrate the critical role played by IL-12 in the rejection of male skin graft by female C57BL/6 syngeneic recipients and in the clearance of an immunogenic mastocytoma tumor variant by DBA/2 mice, but not in a parent to F1 immune aggression model nor in MOG-induced EAE, which was clearly prevented by anti-p40 mAb C17.8. Given this selective inhibition of IL-12, these mAbs provide new options for reassessing IL-12 function *in vivo*.

Keywords: EAE · IL-12 · Sepsis · Transplantation · Tumor immunity

Additional supporting information may be found online in the Supporting Information section at the end of the article.

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Introduction

IL-12 is a major promoter of T_H1 differentiation and IFN-γ production involved in the control of innate and adaptive immunity [1–3]. It is required for proper control of a number of infections such as *Leishmania major* [4], *Toxoplasma gondii* [5], *Mycobacterium tuberculosis* [6, 7] and *Listeria* [8]. Moreover, it contributes to anti-tumor immunity by stimulating CTL and NK cells [9–12]. However, IL-12 also contributes to numerous infectious and auto-immune pathologies like Schwartzman reaction [13], psoriasis [14], myasthenia gravis [15], glomerulonephritis [16], and nephropathy [17]. It is obvious, therefore, that selective blocking of IL-12 activity could have significant therapeutic potential.

Specific inhibition of IL-12 with Abs is a complex task since its two subunits, p40 and p35, can each associate with other partners. p40 combination with p19 yields IL-23, which is a key player in auto-immunity [18], and p35 association with Ebi3, a member of the p40 family, forms IL-35 [19], which is involved in Treg inhibitory function [20] and was reported to mediate control of EAE by B cells [21]. As a result of these complex interactions, IL-12 was originally claimed to contribute to EAE based on inhibition by anti-IL-12 p40 Abs [22], while the actual culprit was later identified as IL-23 [23].

Previously, we described an IL-12 auto-vaccine produced by directly conjugating mouse IL-12 to OVA or to a Pan-DR epitope (PADRE) peptide by incubating each component with glutaraldehyde [24]. This vaccine, which induced mostly anti-p40 Abs and thus inhibited both IL-12 and IL-23, prevented EAE and also atherosclerosis [24, 25] at the expense of decreased resistance to *Leishmania major* infection [24]. Moreover, this vaccine exacerbated susceptibility of naive mice to *Mycobacterium tuberculosis* [26], further illustrating the power of auto-vaccination as a tool for *in vivo* analysis of cytokine function as initially promoted for IFN-alpha by Zagury et al. [27].

In an attempt to produce a more specific anti-IL-12 vaccine, we used a procedure based on the reaction of IL-12 with glutaraldehyde-covered OVA polymers avoiding exposure of IL-12 to free glutaraldehyde as in our previous vaccine. This procedure has already allowed for the production of Abs neutralizing TGF-ß1, GM-CSF, IL-17F, and IL-27 [28]. We here report the induction and characterization of mAbs that exclusively react with the IL-12 p35-p40 heterodimer, not with p40 or p35, and their use to re-evaluate the pathologic contribution of IL-12 in various immune-mediated diseases.

Results

Anti-IL-12 auto-vaccination and production of Abs specifically recognizing IL-12 heterodimer

B6 mice were immunized with mouse IL-12 conjugated to OVAglu polymers as previously described for other cytokines [28]. After five immunizations with the two forms of IL-12-OVA complexes described in Materials and Methods (1/1 and 1/4 IL-12/OVA molar ratios), equivalent Ab serum titers, in the range of 10^{-5}, were detected by ELISA on IL-12-coated ELISA plates (data not shown). Seven mAbs, all IgG2a-k, derived from several selected mice were compared for binding to biotinylated IL-12 adsorbed on avidin-coated plates. This procedure was used to avoid epitope alterations after direct binding of IL-12 to polystyrene. Titrations of these mAbs (Fig. 1A) indicated that they all had stronger or equivalent binding than C17.8 the reference rat anti-mouse p40 mAb [29] and MMp35.8G7, a mAb produced from mice vaccinated with IL-12p35 N-terminal peptide–OVAglu complexes [30]. Avidities calculated on the basis of half-maximal binding were on the order of 0.1–0.6 nM, which compared favorably to C17.8 (± 2 nM).

A first evaluation of the specificity of these mAbs was performed by coating the Abs on ELISA plates and measuring capture of biotinylated IL-12, p40, IL-35, and IL-23. All mAbs strongly bound IL-12 but not p40, contrary to C17.8 that bound both (Fig. 1B). They also failed to significantly capture IL-35 unlike MMp35.8G7 that captured IL-12 and IL-35 equivalently and they were unable to bind IL-23 unlike C17.8 mAb. The quality of the biotinylated IL-35 was further ascertained by its binding to anti-Ebi3 mAb MEBI3B1. The lack of interaction of MM12 mAbs with IL-35 was confirmed in IP and western blot experiments as already reported previously for MM12A1.6 [30]. As shown in Supporting Information Fig. S1A and B, p40 was detected in western blots following IP of IL-12 supernatants with all MM12 mAbs, however none of the mAbs immunoprecipitated IL-35 at levels detectable by western blot with anti-Ebi3. These results were further corroborated by assessing binding of the mAbs to yeast cells expressing single-chain constructs of IL-12 or IL-35 using the yeast surface display system. All of the mAbs bound to IL-12 on the yeast cell surface, while no detectable binding to IL-35-expressing yeast was observed (Supporting Information Figs. S1C and S2). The inhibitory activity of the mAbs was tested using Ba/F3 pro-B cells transfected with IL-12R as previously described [24]. Ba/F3 cells are highly dependent on IL-3 for growth and survival but, after transfection with IL-12 receptor, they show short-term proliferation in response to IL-12. Figure 2A shows the results for one of the hybridomas, MM12A1.6, that completely inhibited proliferation of Ba/F3 cells induced by IL-12. Of the seven hybridomas tested, five were inhibitory with IC50 comparable to that of C17.8. The two mAbs that were nonblocking were equivalent to the blocking ones with respect to IL-12 binding avidity, suggesting that they recognized distinct epitopes. This was confirmed when mAbs were compared to each other in competition for capture of biotinylated IL-12: the four inhibitory mAbs tested competed with each other not with the two noninhibitory ones and reciprocally (Fig. 2B), demonstrating that blocking and nonblocking Abs interacted with nonoverlapping IL-12 epitopes.

The present IL-12-OVAglu conjugates thus induced Abs reacting specifically with a limited number of epitopes present in the p35-p40 IL-12 heterodimer not in IL-35 (p35-Ebi3) nor in p40 monomers, contrary to our previous vaccine that induced mainly anti-p40 Abs [24]. A somewhat unfortunate consequence of these findings is that the name MMp35A1.6 that we previously used for

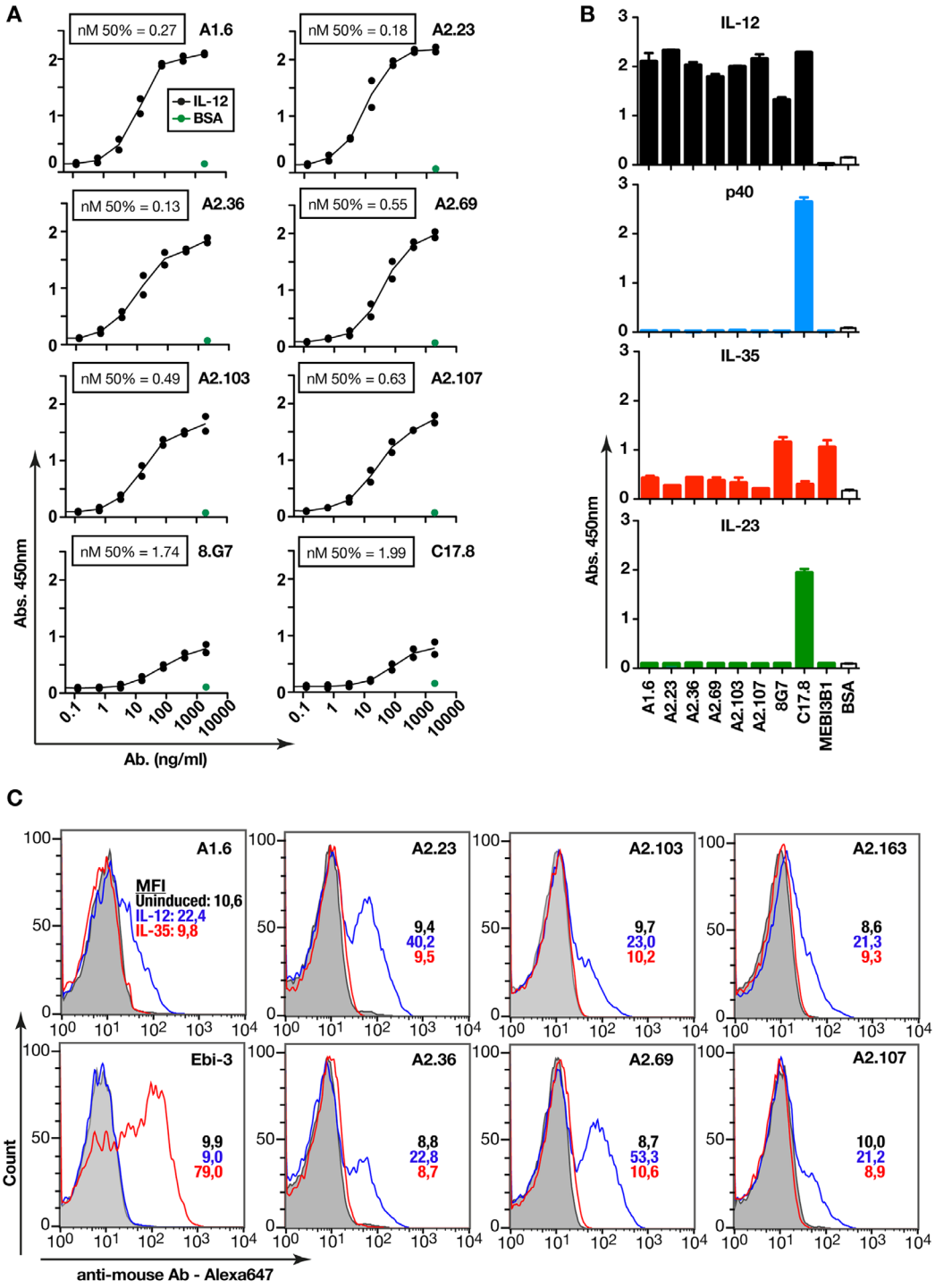
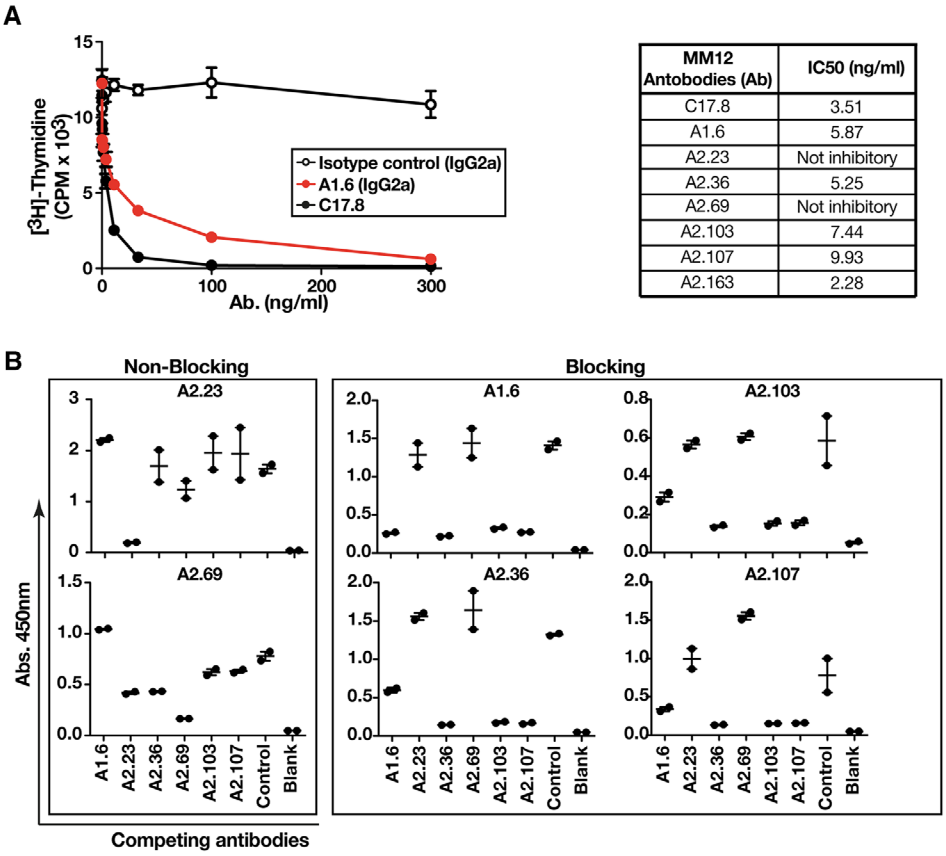


Figure 1. MM12 Abs specifically recognize IL-12p35-p40 but not IL-35, p40, or IL-23. (A) B6 mice immunized with mouse IL-12 conjugated to OVAglu polymers were used to produce anti-IL-12 hybridomas that were designated MM12. Binding of IL-12 to six such mAbs (A1.6, A2.23, A2.36, A2.69, A2.103, and A2.107), anti-IL-12p35 Nter peptide Ab MMp35.8G7 (8G7), and anti-p40 Ab C17.8 is shown here. Briefly, ELISA plates coated with avidin (5 μg/mL) or BSA were incubated with biotinylated IL-12 at 200 ng/mL for 1 h, washed and then incubated with various concentrations of Abs for 2 h. Bound Ab was detected with HRP-conjugated goat anti-mouse Ig followed by TMB and absorbance reading at 450 nm. nM concentrations for half- maximal binding are indicated (nM 50%). Data are representative of two independent experiments with technical duplicates per experiment. (B) Binding of biotinylated IL-12, p40, IL-35 (p28-human Ig-Ebi3), and IL-23, all at 200 ng/mL, to immobilized Abs or BSA detected with avidin-HRP (mean of triplicates ± SEM). Data are representative of two independent experiments with technical triplicates per experiment, where IL-35 was detected with goat anti-human IgG-HRP. (C) Single-chain constructs of IL-12 (p40-linker-p35) and IL-35 (Ebi3-linker-p35) were cloned into pCT302 yeast display vector before being transformed into *S. cerevisiae* yeast display strain EBY100. Yeast cells expressing IL-12 or IL-35 were incubated with the indicated Ab followed by anti-mouse Alexa 647 and flow cytometry analysis. Uninduced IL-12 yeast grown in the absence of galactose and lacking surface expression were stained as a negative control. Data representative of two experiments performed in monoplicate.



MM12 Antobodies (Ab)	IC50 (ng/ml)
C17.8	3.51
A1.6	5.87
A2.23	Not inhibitory
A2.36	5.25
A2.69	Not inhibitory
A2.103	7.44
A2.107	9.93
A2.163	2.28

Figure 2. MM12 mAbs recognize a limited number of epitopes on IL-12p35-p40 heterodimer that correlate with their inhibitory activity. (A) B6 mice immunized with mouse IL-12 conjugated to OVAglu polymers were used to produce anti-IL-12 hybridomas that were designated MM12. Inhibition of IL-12-induced proliferation of Ba/F3 mIL-12R cells by MM12 Abs, C17.8, and a control IgG2a by serial dilutions of the Abs and representative of two distinct experiments. Mean ± SD are computed from technical duplicate in each experiment. IC50 calculated for each Ab are indicated. (B) Competition of MM12 Ab for binding to IL-12: biotinylated IL-12 (200 ng/mL) was incubated with 20 μg/mL of each Ab overnight before transferring the mix to ELISA plates coated with the different MM12 Abs. The positive control (Control) is a well with coated Ab + biotinylated IL-12 only and negative control (Blank) is a well without coated Ab and incubated with the mix of biotinylated IL-12 + mAbs. After 1 h incubation at 37°C and washing, plate-bound biotinylated IL-12 was measured by 1 h incubation with avidin-HRP followed by TMB. The results correspond to duplicate samples and are representative of two independent experiments.

A1.6 because this mAb reacted with IL-12 not with p40 [30] is misleading. As from now, we will refer to this mAb as MM12A1.6.

MM12A1.6 inhibits viral-induced IFN-γ production and modulates septic shock

To evaluate the activity of MM12A1.6 *in vivo*, we used infection with a nidovirus, lactate dehydrogenase-elevating virus (LDV), which has been shown to dramatically enhance susceptibility to septic shock induced by LPS especially in *IFNAR-1*$^{-/-}$ mice, where increased mortality correlates with massive production of IFN-γ and IL-12 [31]. Using the protocol depicted in Fig. 3A, we first tested the role of IL-12 in the induction of IFN-γ production by LDV infection in both WT and *IFNAR-1*$^{-/-}$ 129/Sv mice. IFN-γ was strongly upregulated in both strains by the infection particularly in *IFNAR-1*$^{-/-}$ mice where its concentration peaked at 895 ± 49 pg/ml (mean ± SEM) as compared to 302 ± 95 pg/ml in WT mice. In both strains, MM12A1.6 completely inhibited this IFN-γ production, demonstrating the *in vivo* efficacy of the Ab and the essential role of IL-12 in this viral-induced IFN-γ production even in the absence of Type-1 IFN signaling (Fig. 3B). Given the importance of IFN-γ as a mediator of septic shock induced by the LDV-LPS combination [31] and the above-mentioned importance of IL-12 in IFN-γ induction by LDV, we tested the role of IL-12 in the lethality induced by LPS in LDV-infected mice. MM12A1.6 was administered one day before infection with LDV which was followed 24 h later with 10 μg LPS. In *IFNAR-1*$^{-/-}$ mice, all control mice died after 72 h but all survived in the MM12A1.6-treated group. In WT mice, death occurred earlier than in *IFNAR-1*$^{-/-}$ mice and here MM12A1.6 did not save the mice, although it significantly increased mean survival time from 25 to 110 h (Fig. 3C).

MM12A1.6 establishes IL-12 role in tumor and skin graft rejection but not in parent to F1 disease

Immunogenic variants of P815 mastocytoma tumor cells that are spontaneously rejected by DBA/2 mice have been obtained by incubating these tumor cells with mutagen N-methyl-N'-nitro-N-nitrosoguanidine *in vitro* [32] and IL-12 has been suggested to contribute to this tumor rejection process [33]. In the published experiments, IL-12 was inhibited with a rabbit serum produced prior to identification of cytokines sharing IL-12 subunits. To formally determine the requirement of IL-12 in this rejection process, we used MM12A1.6 and compared it to anti-p40 mAb C17.8. Mice were injected with P911 cells, an immunogenic variant of P815, and treated with 0.5 mg mAb every week for 2 months (Fig. 4A). As shown in Fig. 4B, these tumor cells killed all irradiated (6 Gy) immune suppressed mice in less than 3 weeks but none of the nonirradiated control mice, confirming the importance of the immune control of these tumor cells, which are eliminated by CD8^{+} cytolytic T cells [34]. In contrast, all MM12A1.6-treated mice died, but with a significant delay as compared to

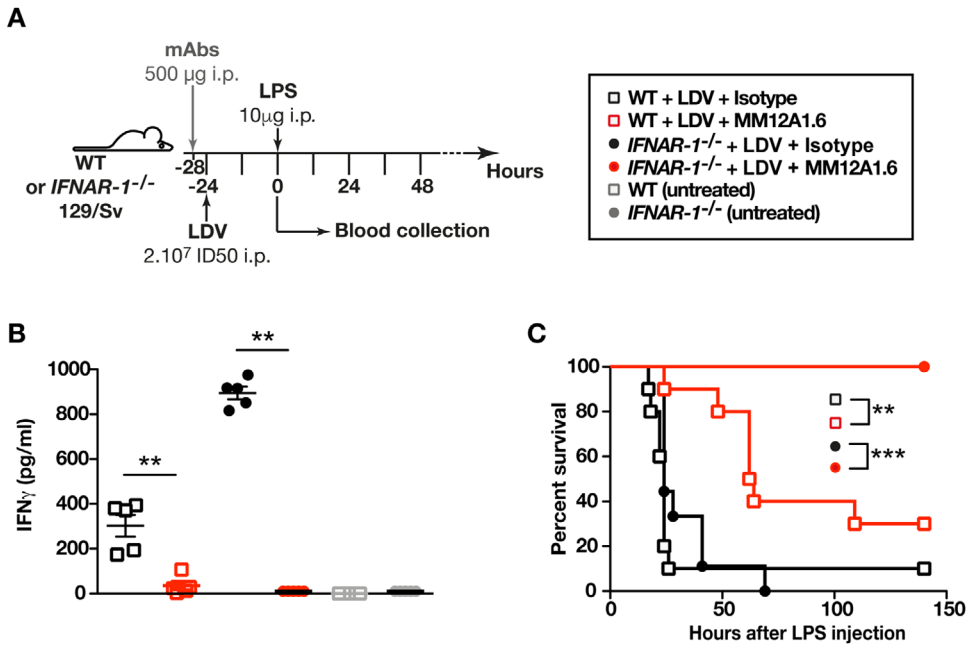


Figure 3. MM12A1.6 prevents viral-induced IFN-γ production and impairs LPS-induced septic shock in *IFNAR-1*$^{-/-}$ and WT 129/Sv mice. (A) Schematic representation illustrates the experimental protocol. *IFNAR-1*$^{-/-}$ and WT 129/Sv mice were injected or not with 0.5 mg MM12A1.6 mAb or control IgG2a AH9R3A mAb i.p. 4 h before infection with LDV. (B) Plasma IFN-γ concentrations were measured by ELISA 24 h after infection (five mice per group). Data are representative of two experiments. Each data point represents the value measured in individual mice with $n = 5$ for LDV + isotype – WT; $n = 6$ for LDV + MM12A1.6 – WT; $n = 5$ for LDV + isotype – *IFNAR-1*$^{-/-}$; $n = 5$ for LDV + MM12A1.6 – *IFNAR-1*$^{-/-}$, $n = 4$ for untreated WT and $n = 5$ for untreated IFNAR-1$^{-/-}$. Difference between MM12A1.6 and control LDV was calculated by Mann–Whitney unpaired *t*-test (**$p < 0.01$) and error bars represent mean ± SEM. (C) The same experiment was performed as in B but 10 µg LPS was administered 24 h after LDV and survival was monitored. Overall survivals of two experiments are depicted with a total of ten mice for LDV + isotype – WT; ten mice for LDV + MM12A1.6 – WT; nine mice for LDV + isotype – *IFNAR-1*$^{-/-}$; ten mice for LDV + MM12A1.6 – *IFNAR-1*$^{-/-}$ (**$p < 0.01$ and ***$p < 0.001$ by the log-rank test).

irradiated recipients, formally establishing the contribution of IL-12 in the tumor rejection process. Similar results were obtained with C17.8 anti-p40 mAb, indicating that MM12A1.6 is as potent as C17.8 to block IL-12 activity *in vivo*.

Local CD8$^+$ T-cell-mediated immune response is also implicated in the rejection of H-Y-disparate skin transplantation [35]. To the best of our knowledge, the role of IL-12 in this skin transplantation has not been studied with IL-12-specific inhibitors. To address this question, we used B6 male skin cells that express the H-Y antigen and are rejected by B6 female mice [36]. MM12A1.6 was injected once a week (0.5 mg) over a period of 175 days (Fig. 5A). In the control group, all male skin grafts were rejected by day 68 (mean survival time: 42 ± 15 days) and all female grafts survived. In the anti-IL-12-treated mice, two of the nine male grafts survived permanently (more than 180 days) and the survival time of the other transplants was 70 ± 23 days ($p = 0.004$ *t* test as compared to controls) (Fig. 5B).

Another graft model frequently studied is parent to F1 hematopoietic cell transplantation that, in some combinations, can induce BM failure and represents a model for aplastic anemia [37]. When B6 parental spleen cells are injected into nonconditioned B6D2F1 recipients, allo-antigen-reactive donor T cells mount a T_H1 reaction leading to acute lethality with massive lymphoid cell destruction contrary to the chronic auto-immune lupus-like T_H2 syndrome that develops when D2 spleen cells are transplanted [38]. To test the importance of IL-12 in the T_H1 reaction seen in the B6 to B6D2F1 spleen cell transplantation model, we administered MM12A1.6 or control mouse IgG2a mAb and observed a small trend for slower weight loss in the anti-IL-12-treated group between day 11 to 15 (Fig. 5D) but all mice died after 18 days (Fig. 5E). In agreement with these clinical data, FACS analysis of blood cells at day 11, showed unimpaired B6 cell implantation as 558 ± 191 (mean ± SEM) CD4 cells and 3447 ± 964 CD8 cells per mm^3 were counted in blood of mice treated with MM12A1.6 as compared to 324 and 989, respectively, in normal mice and almost complete destruction of host cells (Supporting Information Fig. S3A and B). No inhibition of IFN-γ secretion, which is an important inflammatory cytokine in this model, was observed (Supporting Information Fig. S3C). These results were surprising since goat anti-IL-12 Abs have been reported to prevent disease in the same model [39]. We therefore repeated the experiments but also included C17.8 anti-p40 inhibitory mAb. The latter significantly protected B6D2F1 recipients but MM12A1.6 again failed to do so (Fig. 5F).

MM12A1.6 does not prevent EAE

Previous work has suggested the implication of IL-12 in EAE [40] and our own experiments showed that an anti-IL-12 auto-vaccine that induced mainly anti-p40 Abs impaired EAE pathology [24]. Subsequently it was, however, demonstrated that IL-23, not IL-12,

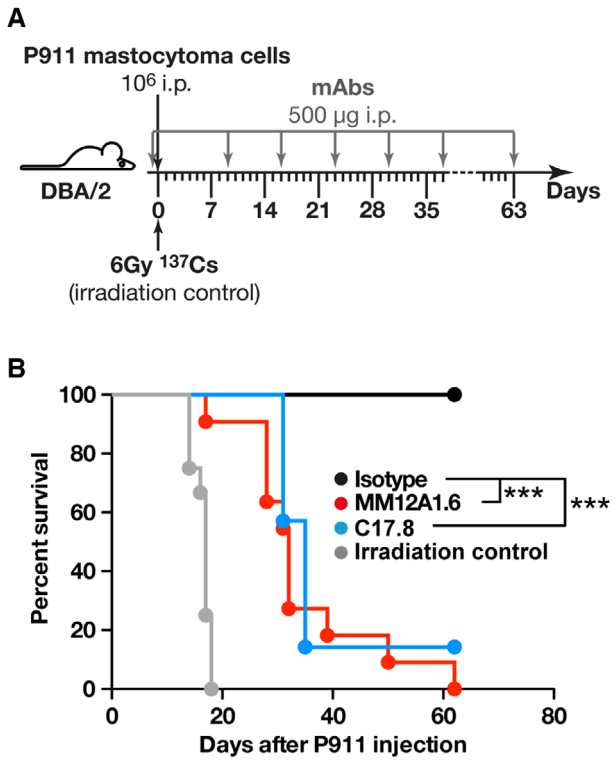


Figure 4. MM12A1.6 impairs rejection of an immunogenic mouse P815 tumor variant. (A) Schematic representation illustrates the experimental protocol. Female DBA/2 mice were injected i.p. with 10^6 tum$^-$ P911 cells and once a week with 0.5 mg MM12A1.6, C17.8, or control IgG2a AH9R3A mAb. To verify the immunogenic nature of P911 rejection in control mice, a group was irradiated with 6 Gy from a ^{137}Cs source. (B) Mice were monitored daily and overall survivals from two independent experiments are depicted with 11 mice for isotype group; 11 mice for MM12A1.6 group; seven mice for C17.8 group and 12 mice for irradiation control group (***$p < 0.001$ by the log-rank test).

is the critical contributor to EAE pathology [23, 41] and IL-12p35-deficient mice showed normal disease severity [42]. Moreover, administration of a monomeric form of IL-12p35 was recently shown to attenuate MOG-induced pathology [43]. Given these discordant observations, it was of interest to compare, in the same MOG-induced EAE experiment, specific IL-12 inhibition by MM12A1.6 and p40 inhibition by C17.8 (Fig. 6A). As shown in Fig. 6B and C, MM12A1.6 did not diminish EAE scores or morbidity as assessed by weight loss unlike C17.8 that completely prevented disease. Lack of EAE prevention by MM12A1.6 was also observed in proteolipid protein (PLP 139–151)-induced disease in SJL mice (data not shown).

Discussion

Ab-mediated inhibition of cytokines is a powerful tool to study the function of these factors *in vivo* and *in vitro*. However, this task is extremely complicated for heterodimeric molecules like IL-12. Specific inhibition of this cytokine requires Abs that do not react with other factors sharing either p40 (IL-23) or p35 (IL-35) subunits. This is a major challenge and, to the best of our knowledge, only one mAb with this specificity, produced in the rat, has been described but it has not been tested for IL-35 binding nor used to reevaluate multiple functions of IL-12 *in vivo* [44]. We here reported the characterization of mouse Abs that specifically recognize and inhibit the mouse IL-12 heterodimer. By using our previously described OVAglu amine-reactive polymers [28] as a carrier and recombinant IL-12 produced as a natural heterodimer in mouse P815 cells, we obtained a vaccine that induced mouse Abs inhibiting IL-12 but that failed to react with p40 and IL-35, which excluded their interaction with p35-restricted epitopes. In previous experiments, where IL-12 and OVA were directly conjugated through incubation with glutaraldehyde, we mainly obtained anti-p40 Abs that inhibited EAE in agreement with the predominant role played by IL-23 in this disease [45]. The reason for the difference could be related to the higher preservation of the natural configuration of IL-12 with the OVAglu polymers as suggested by the significant morbidity that we observed after vaccine administration (C.U. personal observation), in accordance with the well-known toxicity of this cytokine [46].

The IL-12 specificity of the anti-IL-12 mAbs produced here is intriguing. The Abs were unable to bind p40 monomers and therefore failed to inhibit IL-23 bioactivity but also did not react with IL-35, which includes p35. This suggests that these Abs react with conformational domains present only in IL-12 as they fail to react with IL-35 and reduced-alkylated IL-12 (unpublished observation, JVS).

Infection of mice with various RNA viruses strongly enhances susceptibility to septic shock induced by LPS [31, 47, 48]. The role of IL-12 in this process has not been clarified. Our results, using LDV as an RNA virus model, show that, both in WT and in *IFNAR-1*$^{-/-}$ mice, MM12A1.6 strongly suppressed IFN-γ production after infection demonstrating the essential role of IL-12, not type I IFNs, in this IFN-γ upregulation, which in this system is mainly produced by NK cells [49]. Type I IFNs, however, contributed to mortality induced by LPS challenge of LDV-infected mice as MM12A1.6 only delayed mortality in 129/Sv WT mice while it completely and permanently protected 129/Sv *IFNAR-1*$^{-/-}$ mice. These observations demonstrated the importance of IL-12 in this pathology and also the efficacy of MM12A1.6 as a tool for evaluating the role of IL-12 in pathological conditions *in vivo*.

The implication of IL-12 in the antitumor immune response elicited in DBA/2 mice against P815 tumor-minus mastocytoma variants has been evaluated in previous reports but with reagents lacking strict IL-12 specificity. Here, we observed that MM12A1.6 was as potent as C17.8 in impairing rejection of P911 tumor-minus variant, formally proving the essential contribution of IL-12 to this CD8$^+$ T-cell-mediated tumor control. Of note, sublethal mouse irradiation resulted in faster death than MM12A1.6 treatment, indicating that IL-12 is not the only factor contributing to the immune control of tumor-minus P911 variant growth *in vivo*. IL-12 was, however, essential for maintaining long-term immune protection since all IL-12-deprived mice ultimately died, in agreement with the ability of IL-12 to enhance CTL

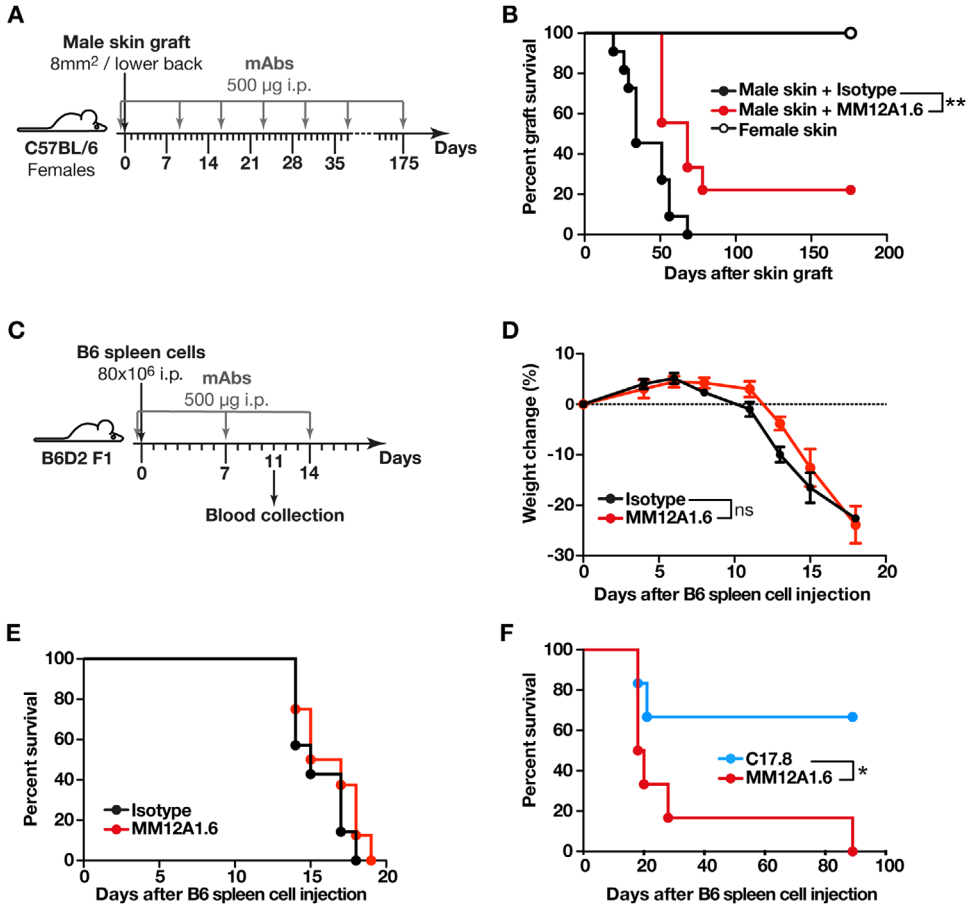


Figure 5. MM12A1.6 improves skin graft survival but does not prevent parent to F1 immune aggression model. (A) Schematic representation of the skin graft protocol. Male B6 skins were grafted on female B6 mice that were treated with MM12A1.6 or control IgG2a AH9R3A mAb (0.5 mg Ab every week throughout the duration of the experiment). As a control, female skin grafts were included. (B) Graft rejection was scored daily. One representative experiment of two is shown with 10–11 mice for male skin + isotype group; nine to ten mice for male skin + MM12A1.6 group; four to seven mice for female skin group per experiment (**$p < 0.01$ by log-rank test). (C) Schematic representation of the parent to F1 spleen cell transfer protocol. B6D2F1 mice were injected i.p. with 80 million B6 spleen cells and treated with MM12A1.6 or control IgG2a AH9R3A mAb (0.5 mg) i.p. just before spleen cell transfer and every 7 days subsequently. Weight loss (D) and survival (E) were monitored daily. One representative experiment of three is shown with five to seven mice for isotype group and six to eight mice for MM12A1.6 group per experiment. Statistical analysis was performed with ANOVA–Bonferroni posttest and log-rank test and error bars of weight change graph represent mean ± SEM. (F) B6D2F1 mice were injected i.p. with 80 million B6 spleen cells and treated with MM12A1.6 or C17.8 mAb (0.5 mg) i.p. just before spleen cell transfer and every 7 days subsequently. Survival was monitored daily. One representative experiment of two is shown with five to six mice for C17.8 group and five to six mice for MM12A1.6 group per experiment. Statistical analysis was performed with log-rank test (*$p < 0.05$).

activity against another peptide of P815 tumor-minus variant cells [9].

The role of IL-12 in the rejection of skin and cardiac allografts has been studied with anti-p40 Abs or polyclonal anti-IL-12 Abs with discordant results. Piccotti reported that mice treated with anti-p40 Ab or with a p40 homodimer used as an IL-12 antagonist showed normal or even accelerated allograft rejection [50]. In contrast, anti-p40 Ab was found to delay rejection of mouse cardiac allograft [51] and anti-IL-12 treatment combined with a previous injection of donor lymphoid cells also prolonged graft survival [52]. The anti-IL-12 Abs used in these experiments could also have affected IL-23 or IL-35 activities, thus giving opposite signals for effector T-cell differentiation. Our results now demonstrate that specific inhibition of IL-12, even without donor spleen cell transfusion, is sufficient to extend graft persistence in male to female C57BL/6 skin transplantation, a model where 100% of the grafts are normally rejected. This observation formally establishes that IL-12 is a critical control point in skin transplantation in line with the fact that administration of IL-12 induces rejection of male skin graft in female CBA mice where these grafts are normally tolerated [53]. A role of IL-12 in graft-versus-host disease (GvHD) was first suggested by observations showing inhibition of the disease by goat anti-IL-12 antisera in a B6 to nonconditioned B6D2F1 recipient model [54], which is now mainly considered as an aplastic anemia model [37]. This idea was also supported by results obtained in GvHD experiments that used conditioned IL-12p40-deficient B6 recipients of FVB mouse donor cells and in the human with ustekinumab, a humanized anti-p40 Ab [55]. To start assessing the effect of specific inhibition of IL-12 with MM12A1.6 mAb in this disease model, we used B6 spleen cell transplantation

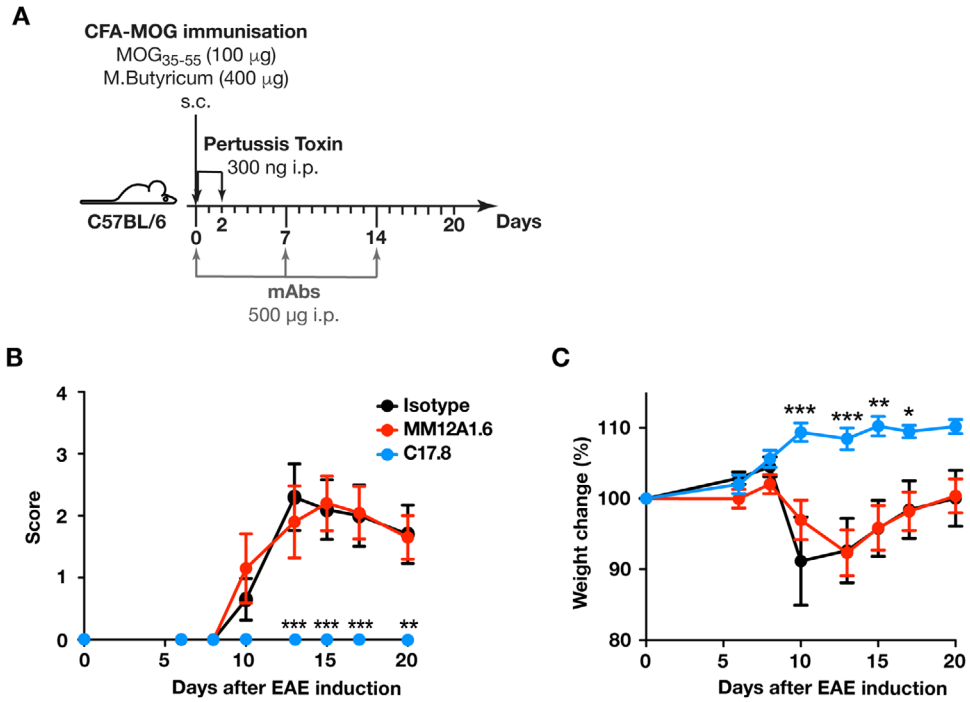


Figure 6. MM12A1.6 does not prevent EAE. (A) Schematic representation of EAE induction and Ab treatment. C57BL/6 mice were injected with 100 μg myelin oligodendrocyte glycoprotein (MOG) 33–55 peptide in CFA containing 400 μg *Mycobacterium butyricum*. On days 0 and 2, mice received 300 ng *Bordetella pertussis* toxin i.p. MM12A1.6 and C17.8 injection times (0.5 mg i.p.) are indicated by arrows. (B) EAE scores and (C) weight loss were monitored twice a week in one experiment with nine mice for isotype group; 11 mice for MM12A1.6 group and ten mice for C17.8 group. Statistical analysis by two-way ANOVA (***$p < 0.001$, **$p < 0.01$, *$p < 0.05$ and error bars represent mean ± SEM. Lack of protection by MM12A1.6 was observed in a second experiment with six mice for isotype group; six mice for MM12A1.6 group and seven mice for C17.8 group

into nonconditioned B6D2F1 recipients because this protocol induces a milder disease that is easier to inhibit than that induced in irradiated recipients of fully allogeneic T and BM cells as we experienced during our studies that showed the role of IL-27 in this pathology [56, 57]. Surprisingly, contrary to anti-IL-27p28 mAb, MM12A1.6 failed to protect and did not block IFN-γ synthesis in these mice while it did so in experiments with viral infection. The mAb also failed to confer protection in B6 to B6D2F1 experiments where C17.8 rat anti-p40 mAb enhanced survival. A possible explanation would be that in this experimental model, IL-23 (p40-p19), which is also blocked by anti-p40 Abs but not by MM12A1.6, is the critical factor in line with its reported contribution to some forms of GvHD [58, 59]. In any case, these results are puzzling and will need to be confirmed in transplantation models involving irradiated recipients to confirm these results in *bona fide* GvHD models to avoid misleading conclusions. Nevertheless, the present results show that, contrary to IL-27, IL-12 is not critical for IFN-γ production during B6 to B6D2F1 transplantation, a point that was already reported in the less inflammatory model of cardiac allograft [60].

Finally, the failure of MM12A1.6, as opposed to C17.8, to prevent MOG-induced EAE in B6 mice confirmed the fact that IL-12 is not important in this model, where disease was completely prevented by anti-p40 Abs, in line with the predominant role of IL-23 in this disease [61]. Moreover, the unchanged recovery observed in MM12A1.6-treated mice provides further evidence that this Ab does not interfere with IL-35 activity *in vivo* since the latter cytokine is required for recovery of normal brain function after encephalitis [21].

Together, our results describe the production of anti-mouse IL-12 Abs specifically blocking the p35-p40 heterodimer without affecting p40 and IL-35 functions, thus providing a unique tool to redefine the function of this cytokine *in vivo*.

Materials and methods

Mice

C57BL/6 (B6), DBA/2, *IFN-α/ßR*$^{-/-}$ (*IFNAR-1*$^{-/-}$) [62], and WT 129/Sv mice were bred under specific pathogen-free conditions at the animal facility of the Ludwig Cancer Research Brussels Branch under the direction of Guy Warnier (DVM). Experimental protocols and animal handling were approved by the ethical committee of the Medical Faculty of the Université de Louvain (accreditation: 2016/UCL/MD/010).

Ab preparation for in vivo use

All Abs were produced *in vitro* by culturing hybridomas in Hybridoma-SFM medium (Thermo Fisher Scientific, Be) and

purified on HiTrap Protein A columns according to manufacturer instructions (GE Healthcare, Se). LPS contamination was eliminated by passage on Sartobind-SP cartridges (Sartorius, De).

Production of IL-12-OVA conjugates

His-tagged mouse IL-12 produced in P815 mastocytoma cells [33] (232 μg) was conjugated to glutaraldehyde-activated OVA polymers (OVAglu) as previously described for other factors [28] in 1/1 or 1/4 IL-12/OVA molar ratios. PADRE peptide (CKXVAAWTLKAAZ, where X = cycloexylamine) was added to saturate remaining free glutaraldehyde sites. For immunization, C57Bl/6 mice were treated with 0.5 mg PC61 anti-CD25 Ab for Treg depletion 2 days before immunization with 5 μg IL-12-OVAglu-PADRE complexes emulsified in Gerbu 100 adjuvant (Gerbu, Gaiberg, Ge). Immunizations were performed in the footpads and repeated five times at 2-week intervals.

Detection and specificity analysis of anti-IL-12 Abs by ELISA

Sera of vaccinated mice were tested for anti-IL-12 Abs by ELISA on IL-12 (500 ng/mL in 40 mM glycine buffer pH 9)-coated Maxisorb ELISA plates (Nunc, UK) as described [24]. Bound Abs were detected with goat-anti-mouse IgG-HRP (Sigma-Aldrich) followed by Ultra-TMB substrate (Thermo Fisher Scientific, Be).

For detection and analysis of anti-IL-12 mAbs, biotinylated IL-12 (sulfo-NHS-biotin; Thermo Fisher Scientific, Be) was bound to avidin-coated (5 μg/mL; Sigma-Aldrich) ELISA plates and incubated with Abs followed with goat anti-mouse IgG-HRP. To determine IL-12 subunit specificity, ELISA plates were coated with the purified mAbs followed by incubation with biotinylated IL-12, p40 (both home-made as described above), or IL-35 (Chimerigen Laboratories, Ca) at 200 ng/mL. Bound cytokines were detected with avidin-HRP (Biolegend, Ca). A mouse anti-Ebi3 specific mAb MEBI3.B1 produced in house, MMp35.8G7, a mAb specific for p35 N-terminal peptide previously described [30], and AH9R3A anti-human IL-9 receptor (IgG2a) were included as controls. In Supporting Information Fig. S4, the Ebi3 specificity of MEBI3.B1 (1 μg/mL) is shown on triplicate ELISA wells directly coated with mouse Ebi3, IL-27 (p28-Ebi3), and IL-12p28 (gift of Mike Jones, Shenandoah Biotechnology, MD, USA) at 500 ng/mL. Bound Ab was detected as described above.

Assays of anti-IL-12 Ab interaction with IL-12 and IL-35

Culture supernatants from transient transfection of HEK293T cells with native IL-12, native IL-35, or empty pCIneo vector were generated as previously described [28] for western blot and IP.

For western blots, vector control or IL-35 supernatant samples were reduced by boiling in sample loading buffer containing 10% β-mercaptoethanol and run on SDS-PAGE. Gels were transferred to nitrocellulose membranes and blocked with Tris buffered saline (TBS) containing 0.002% Tween-20 (TBS-T) and 3% BSA. Blots were probed with the indicated MM12 Abs or anti-Ebi3 (1 μg/mL), followed by anti-mouse IgG-HRP. Blots were washed and proteins were detected with the Western Lightening ECL system.

IL-12 or IL-35 supernatants were immunoprecipitated overnight with 0.5 μg of the indicated MM12 Abs plus protein G agarose. Agarose was washed with PBS and samples were processed as above for SDS PAGE and western blot. Blots were probed with either biotinylated anti-p40 (IL-12) or biotinylated anti-Ebi3 (IL-35), followed by streptavidin-HRP for detection.

For yeast binding assays, single-chain constructs of IL-12 (p40-linker-p35) and IL-35 (Ebi3-linker-p35) were cloned into the pCT302 yeast display vector as N-terminal fusions to the Aga2 yeast agglutinin, with subunits connected by a 16 amino acid (G3S)4 linker. Constructs were transformed into *S. cerevisiae* yeast display strain EBY100 and expressed under a galactose-inducible promoter as previously described [63]. Yeast cells expressing IL-12, IL-35, or mutants of IL-35 with higher yeast surface expression (IL-35 m2 and IL-35 m3) were incubated for 30 min with 5 μg/mL MM12 or anti-Ebi3 Abs in FACS buffer (PBS + 1% BSA), washed, and stained with Alexa 647-labeled anti-mouse IgG. IL-12 yeast grown in media without galactose do not express IL-12 on the surface and were stained as a negative control.

Assay for IL-12 inhibition in vitro

IL-12 bioassay was performed as previously described [25]. Briefly, 10^4 Ba/F3mIL-12R cells were incubated with Ab and 1 ng/mL recombinant IL-12 (R&D Systems, MN, USA) for 24 h and 1μCi ^{3}HT (PerkinElmer, Waltham, Ma.) was then added for another 16-h incubation. Thymidine incorporation was measured with a Topcount NXT counter (PerkinElmer, Wellesley, MA, USA).

Animal experiments

Septic shock was induced in 7- to 9-week-old *IFNAR-1*$^{-/-}$ or WT 129/Sv mice by i.p. injection of 10 μg *E. coli* O11B4 LPS (Sigma-Aldrich) 24 h after infection with LDV (Riley strain; American Type Culture Collection, Rockville, MD, USA). IFN-γ plasma concentration was measured by ELISA (Cytoscreen, Biosource International, Ca.).

For evaluation of the role of IL-12 in rejection of P911 immunogenic mastocytoma tumor-minus variant cells, DBA/2 mice (8-week old) were injected i.p. with 1 million P911 cells grown in vitro as described [32] and treated once a week with 0.5 mg C17.8, MM12A1.6 or AH9R3A, anti-human IL-9R Ab as a control mouse IgG2a. To verify the viability of the injected tumor cells, a group of mice was immunosuppressed by 6 Gy ^{137}Cs irradiation before tumor injection.

C57BL/6 male skin was grafted in 8-week-old female C57BL/6 mice according to the protocol described by Dr. Elisabeth Simpson [36]. MM12A1.6 (0.5 mg) mAb was injected i.p. once a week

starting immediately after transplantation and continued throughout the experiment. For graft evaluation, partially rejected grafts were scored as rejected.

Parent to F1 disease was induced by transfer of 80 million B6 spleen cells into nonconditioned 8-week-old B6D2 F1 mice. Groups of eight mice were injected i.p. with MM12A1.6 or control mouse IgG2a (0.5 mg) just before spleen transplantation and injections were repeated every week. Chimerism in blood was determined by flow cytometry. Blood cells were stained with antibodies against surface markers (H-2D^b, H-2D^d, CD4, and CD8) in the presence of a viability dye (eBioscience) and anti-CD16/32 to block FcγRs using a standard protocol. Analyses were performed on a FACS LSR Fortessa flow cytometer (DIVA, BD Biosciences) and data were computed using the FlowJo software (Tree Star). IFN-γ plasma concentration was measured by ELISA (murine IFN-γ ELISA; R&D Systems, Ca.)

EAE was induced in 8-week-old C57Bl/6 mice by vaccination with MOG 33–55 peptide as previously described [64]. Groups of nine to ten mice were injected i.p. with C17.8, MM12A1.6, and control mouse IgG2a (0.5 mg) just before EAE induction and injections were repeated every week.

Statistical analysis

Statistical analysis was performed with Prism 5 (Graphpad Software, La Jolla, CA) using nonparametric tests (Mann–Whitney), one-way ANOVA (Kruskal–Wallis), two-way ANOVA (Tukey), and log-rank test for survival experiments.

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Conflict of interest: DAAV: Cofounder and stock holder—Novasenta and Tizona; stock holder—Oncorus and Werewolf; patents licensed and royalties—Astellas, BMS; scientific advisory board member—Tizona, Werewolf, and F-Star; consultant—Astellas, BMS, Almirall; research funding—BMS, Astellas, and Novasenta. All other authors declare no commercial or financial conflict of interest.

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Abbreviations: **GvHD**: graft-versus-host disease · **LDV**: lactate dehydrogenase elevating virus

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