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Tobias Volker Lanz

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Dekan: Professor Dr. I. B. Autenrieth

1. Berichterstatter: Professor Dr. M. Platten

2. Berichterstatter: Professor Dr. H. G. Rammensee

3. Berichterstatter: Professor Dr. S. Meurer

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Inhaltsverzeichnis

Mouse mesenchymal stem cells suppress antigen-specific TH-cell immunity independent of indoleamine 2,3-dioxygenase 1 (IDO1)

Tobias V. Lanz[1,2], Christiane A. Opitz[1,2], Peggy P. Ho[3], Ankur Agrawal[3], Christian Lutz[4], Michael Weller[5], Andrew L. Mellor[6], Lawrence Steinman[3], Wolfgang Wick[1], Michael Platten[1]*

[1] Department of Neurooncology, University Hospital of Heidelberg, INF 400, and German Cancer Research Center, INF 280, 69120 Heidelberg, Germany

[2] Department of General Neurology, Hertie Institute for Clinical Brain Research, University of Tübingen, Hoppe-Seyler-Strasse 3, D-72076, Tübingen, Germany

[3] Department of Neurology and Neurological Sciences, Beckman Center for Molecular Medicine, Stanford University, Stanford, CA 94305, USA

[4] Heidelberg-Pharma AG, Ladenburg, Germany

[5] Department of Neurology, University of Zürich, Switzerland

[6] Institute of Molecular Medicine and Genetics, Medical College of Georgia, Augusta, GA 30912, USA

*Address correspondence and reprint requests to Dr. Michael Platten, Department of Neurooncology, University Hospital of Heidelberg, Im Neuenheimer Feld 400, 69120 Heidelberg, Germany, Tel.: +49 6221 56 6804, Fax: +49 6221 56 7554, e-mail: michael.platten@med.uni-heidelberg.de

Abbreviations used: IDO, indoleamine-2,3-dioxygenase; kyn, kynurenine; MOG, myelin oligodendrocyte glycoprotein; MSC, mesenchymal stem cells; TLR, toll-like receptor; trp, tryptophan

Zusammenfassung in deutscher Sprache

Aufgrund ihrer immunsuppressiven Eigenschaften stellen humane mesenchymale Stammzellen (hMSC) ein vielversprechendes Werkzeug dar für zellbasierte Therapien von Autoimmunerkrankungen wie Multipler Sklerose (MS). Bisher wurden vielfach murine MSC (mMSC) eingesetzt um Applikationswege, Migrationsverhalten, zelluläre und molekulare Ziele sowie immunsuppressive Mechanismen von MSC zu erforschen, hauptsächlich in Modell-Autoimmunkrankheiten wie z. B. der experimentellen autoimmunen Enzephalomyelitis (EAE), dem Mausmodell der MS.

Der durch das induzierbare Schlüsselenzym Indolamin-2,3-Dioxygenase 1 (IDO1) vermittelte metabolische Abbau der essentiellen Aminosäure Tryptophan (trp) ist ein wichtiger Stoffwechsel- und Signalweg zur Kontrolle ungewollter Immunreaktionen. Dies geschieht zum einen durch den Entzug von trp und zum anderen durch die Bildung immunsuppressiver Kynurenine (kyn). IDO1 spielt eine wichtige Rolle bei der Unterdrückung von Immunreaktionen durch hMSC. In dieser Arbeit zeigen wir, dass mMSC *in vitro* kein Tryptophan metabolisieren, obwohl IDO1 durch proinflammatorische Stimuli wie Interferon-γ (IFN-γ) oder Toll-like-Rezeptor-Liganden (TLR-Liganden) in mMSC induziert werden kann. Die Abwesenheit dieses immunsuppressiven Stoffwechselweges ist nicht auf defekte TLR-Signalwege zurückzuführen, was wir durch TLR-vermittelte Induktion des Zytokins Interleukin 6 (IL-6) beweisen.

mMSC unterdrücken die Aktivierung antigenspezifischer Myelin-Oligodendrocyte-Glycoprotein (MOG)-reaktiver T-Zellen aus T-Zell-Rezeptor-transgenen Mäusen in der Kokultur. Weder pharmakologische Hemmung noch genetischer Knockout von IDO1 konnte diesen Effekt umkehren. Ebenso führte die systemische Infusion von wildtyp- (wt) und phänotypisch identischen IDO1-defizienten mMSC im EAE-Mausmodell gleichermaßen zu einer Entzündungshemmung sowie zur Verbesserung der klinischen Symptome.

Im Gegensatz zu hMSC zeigen mMSC keine IDO1-vermittelte Unterdrückung antigenspezifischer T-Zell-Antworten. mMSC als Therapeutika in Mausmodellen von Autoimmunkrankheiten sollten mit Vorsicht betrachtet werden im Bezug auf die direkte Übertragung der Ergebnisse auf humane Krankheiten, da sie einen wichtigen immunsuppressiven Signalweg nicht mit hMSC teilen.

Abstract

Due to their immunosuppressive properties human mesenchymal stem cells (hMSC) represent a promising tool for cell-based therapies of autoimmune diseases such as multiple sclerosis (MS). Mouse MSC (mMSC) have been used extensively to characterize and optimize route of administration, motility, cellular targets and immunosuppressive mechanisms in mouse models of autoimmune diseases, such as experimental autoimmune encephalomyelitis (EAE). Tryptophan (trp) catabolism by indolamine-2,3-dioxygenase 1 (IDO1) is a chief endogenous metabolic pathway that tightly regulates unwanted immune responses through depletion of trp and generation of immunosuppressive kynurenines (kyn). IDO1 activity contributes to the immunosuppressive phenotype of hMSC. Here we demonstrate that although IDO1 is inducible in bone marrow-derived mMSC by proinflammatory stimuli such as interferon-γ (IFN-γ) and ligands of toll-like receptors (TLR), it does not lead to catabolism of trp *in vitro*. This failure to catabolize trp is not due to defective TLR signaling as demonstrated by induction of interleukin 6 (IL-6) by TLR activation. While mMSC suppressed the activation of antigen-specific myelin-oligodendrocyte glycoprotein (MOG)-reactive T cell receptor (TCR) transgenic T helper (TH) cells in coculture, neither pharmacologic inhibition nor genetic ablation of IDO1 reversed this suppressive effect. Finally, systemic administration of both, IDO1-proficient and phenotypically identical IDO1-deficient mMSC equally resulted in amelioration of EAE. mMSC, unlike hMSC, do not display IDO1-mediated suppression of antigen-specific T cell responses. mMSC as therapeutic vehicles in mouse models of autoimmune diseases should be used with caution as preclinical models for human disorders as they lack an important immunosuppressive phenotype of hMSC.

1. Introduction

MSC represent a cellular source for tissue regeneration as they can differentiate into multiple cell lineages, such as bone, muscle, cartilage or fat (1). Due to this ability and their capacity to migrate to and to repopulate injured tissues (2), MSC are used in clinical trials to promote muscle and cartilage regeneration. In addition, MSC display a potent immunosuppressive phenotype, which not only enables them to escape rejection when transplanted into allogeneic hosts but also allows them to specifically suppress the host immune response (3). This property has not only sparked clinical trials demonstrating efficacy in graft versus host disease but also expanded research to explore potential application in other inflammatory and autoimmune diseases. For instance, systemic administration of autologous MSC suppresses antigen-specific T cell immunity and subsequent central nervous system (CNS) inflammation in EAE, a mouse model of MS (4-9). MSC regulate multiple cellular components of the immune system such as the maturation and differentiation of antigen-presenting cells (APC), the activation of natural killer (NK) cells and the activation and expansion of CD4+ and CD8+ T cells (10-14). The molecular mechanisms of MSC-mediated immunosuppression appear to involve cell surface molecules and soluble factors (15). The catabolism of the essential amino acid trp by the inducible enzyme IDO1 is involved in the suppression of T cell immunity by hMSC (16-18). IDO1-mediated trp catabolism is a major immunosuppressive effector pathway that inhibits T cell responses to autoantigens and fetal alloantigens (19, 20) and may be instrumental for the therapy of autoimmune diseases such as MS (7, 21-24). Here we address the role of trp catabolism in the suppression of antigen-specific T cell responses by mMSC.

2. Materials and Methods

2.1 Mice

C57BL/6 and SJL mice were purchased from Charles River Laboratories (Sulzfeld, Germany). Transgenic IDO1-deficient (IDO1-/-) mice were generated as previously described (25) and were backcrossed on a C57BL/6 background. 2D2 mice on a C57BL/6 background expressing a T cell receptor (TCR) specific for the MOG peptide p35-55 were kindly provided by Vijay Kuchroo (26). The transgenic TCR in heterozygous mice was detected by flow cytometry of murine blood using antibodies against CD4 and Vβ11 (BD Pharmingen, Heidelberg, Germany). All animal work was performed in accordance with the German animal protection law under the permission of the local authorities in Tübingen and the National Institutes of Health guidelines *Guide for the Care and Use of Laboratory Animals*.

2.2 MSC preparation

MSC were isolated from C57BL/6, IDO1-/- and SJL mice. Femurs and tibiae were removed and cleaned of muscles and connective tissue. Epiphyses were cut off and bone marrow cells were flushed out with D-MEM (HyClone, Logan, UT, USA) using a 23-gauge needle. Cells were centrifuged at 1200 rpm and 4°C for 10 min, suspended in Murine MesenCult® Basal Medium for Mouse Mesenchymal Stem Cells (Stem Cell Technologies, Cologne, Germany) containing 20% Mouse Mesenchymal Stem Cell Stimulatory Supplement (Stem Cell Technologies), 100 U/ml penicillin and 100 µg/ml streptomycin (Cambrex, Charles City, IA, USA) (complete Murine MesenCult® Medium), plated in 75 cm² flasks (Corning, Corning, NY, USA) at a concentration of 5 x 10^5/ml and incubated at 37°C and 5 % CO_2. Medium was changed after 7 days and the adherent cell fraction expanded in complete Murine MesenCult® Medium. MSC were characterized by flow cytometry using a DAKO Cytomation CyanADP Analyzer (DAKO Cytomation, Hamburg, Germany) and DAKO Summit software. The following antibodies were used for FACS analysis: anti-CD11b, anti-CD34, anti-CD44, anti-CD45, (ebioscience, San Diego, CA, USA), anti-CD9, (BD Pharmingen), anti-CD29 (Biolegend, San Diego, CA, USA), anti-CD90 (Cedarlane, Burlington, ONT, Canada), and corresponding isotype controls (ebioscience). Osteogenic differentiation was performed as described (27) with minor variations. Briefly, osteogenic differentiation was induced by adding 0.1 mM dexamethasone, 0.05 mM ascorbic acid, and 10 mM glycerol 2-phosphate disodium salt hydrate (Sigma, Munich, Germany) to complete D-MEM, containing 10 % fetal calf serum (FCS, Biochrom, Berlin, Germany), and 100 U/ml penicillin and 100 µg/ml streptomycin (PAA Laboratories, Pasching, Austria). Cells were plated in this medium in 94 mm tissue culture dishes (Cellstar/Brightpoint, Trier, Germany). Control cells were seeded in complete D-MEM without further supplements. After six weeks of culture calcium deposits were detected by Alizarin Red S (Sigma) staining.

2.3 Dendritic cell (DC) preparation

DC were generated from C57BL/6 and SJL mice. Bone marrow cells were isolated as described above. Cells were suspended in RPMI 1640 (Cambrex), supplemented with 10 % FCS (Biochrom), 100 U/ml penicillin and 100 µg/ml streptomycin (Cambrex) (complete RPMI), and 20 ng/ml (10^6 U/ml) recombinant murine Granulocyte Macrophage Colony Stimulating Factor (rm-GM-CSF) (Immunotools, Friesoythe, Germany). 10^7 bone marrow cells were seeded in 75 cm² flasks (Corning). On day 2, 10 ml of complete RPMI, containing 40 ng/ml rm-GM-CSF, were added. On day 4, cells were washed twice with PBS and detached by incubating cells with 3 ml of accutase for 15 min at 37°C. Cells were centrifuged at 1200 rpm and 4°C for 10 min, then resuspended in complete RPMI containing 20 ng/ml rm-GM-CSF at 5 x 10^5 cells/ml and seeded in 6-well plates with 3 ml/well. On day 6, 3 ml of complete RPMI, supplemented with 40 ng/ml rm-GM-CSF, were added. On day 7 we obtained immature DC, ready to be matured via cytokines or TLR ligands.

2.4 T cell proliferation assay

For T cell proliferation assays spleens were removed and dissociated through a 40 µm cell strainer (BD Falcon, Heidelberg, Germany) to singularize splenocytes and to remove connective tissue, then washed with PBS and centrifuged at 1200 rpm and 4°C for 10 min. Lysis of erythrocytes was performed by suspending the pelleted splenocytes in ACK Lysing Buffer (Cambrex) for one min. Cells were washed twice with PBS and resuspended in complete RPMI. For T cell proliferation assays splenocytes were seeded in 96-well plates (5 x 10^5 cells/well) on 0-5000 mMSCs from C57BL/6 or IDO-/- mice seeded two days before. 24 h earlier mMSCs had been pretreated with 50 µg/ml polyinosinic:polycytidylic acid (pI:C, Sigma), 5 µg/ml lipopolysaccharide (LPS, from *salmonella typhii*, Sigma), or 500 IU/ml IFN-γ (Immunotools). Then mMSC were washed 3 times with PBS to remove all remnants of pI:C, LPS and IFN-γ. Immediately, splenocytes (5 x 10^5 cells/well) of 2D2 mice were added. Activation of T cells was achieved by adding 10 µg/ml MOG p35-55 (MEVGWYRSPFSRVVHLYRNGK, Stanford Protein and Nucleic Acid Biotechnology Facility, Stanford, CA, USA). In some experiments 1 mM 1-methyl-L-tryptophan (1-L-MT) (Sigma) was added immediately after addition of splenocytes. Before harvesting, cultures were pulsed with ^{3}H-methylthymidine (Amersham-Pharmacia Biotech, Munich, Germany) for the last 18 h. Then the 96-well plates were freeze-thawed for three times and harvested using a Tritium Harvester (Tomtec, Unterschleißheim, Germany) and a beta-plate reader (Wallac, Monza, Italy) with BetaWin software. The counts of mMSC proliferation without splenocytes were subtracted from the counts of the cocultures.

2.5 qRT-PCR

RNA was isolated using the Qiagen RNAeasy RNA isolation kit (Qiagen, Hilden, Germany) according to manufacturer's instructions. cDNA was synthesized with the Superscript™ Choice System (Invitrogen, Carlsbad, CA, USA) using random hexamers. qRT-PCR was performed in three serial dilutions, each in duplicates using an ABI 7000 thermal cycler with SYBR Green PCR Mastermix (Eurogentec, Cologne, Germany). Data were evaluated with AB 7000 System SDS software. PCR reactions were checked by including controls without prior reverse transcription and by both melting curve and gel analysis. Relative quantification of gene expression was determined by comparison of threshold values. All results were normalized to b-actin. Primers were designed across exon boundaries. The sequences of the primers were the following: TLR1: fwd: TCAAGCATTTGGACCTCTCCT, rev: TTGTACCCGAGAACCGCTCA, TLR2: fwd: CCAGACACTGGGGGTAACATC, rev: CGGATCGACTTTAGACTTTGGG, TLR3: fwd: GGGGTCCAACTGGAGAACCT, rev: CCGGCGAGAACTCTT-TAAGTGG, TLR4: fwd: ATGGCATGGCTTACACCACC, rev: GAGGCCAATTTTGTCTCCACA, TLR5: fwd: TCAGACGGCAGGATAGCCTTT, rev: AATGGTCAAGTTAGCATACTGGG, TLR6: fwd: GACTCTCCCA-CAACAGGATACG, rev: TCAGGTTGCCAAATTCCTTACAC, TLR7: fwd: TCTTACCCTTACCATCAACCACA, rev: CCCCAGTAGAACAGGTACACA, TLR8: fwd: CAAACAACAGCACCCAAATGAA, rev: AGGCAACCCAGCAGG-TATAGT, TLR9: fwd: ACTCCGACTTCGTCCACCT, rev: GGCTCAATGGT-CATGTGGCA, β-actin: fwd: TGTCCCTGTATGCCTCTGGT, rev: CACG-CACGATTTCCCTCTC; IDO1: fwd: GCTTTGCTCTACCACATCCAC, rev: CAGGCGCTGTAACCTGTGT, KYNA: fwd: AGTGGGCTGCACTTTTATACTG, rev: TGCAAACAGGTTGCCTTTCAG, KMO: fwd: TGATGTGTACGAAGCTAG-GGA, rev: TCATGGGCACACCTTTGGAAA, 3-HAAO: fwd: GGAGGCCCCAATACCAGGA, rev: TATAGGCACGTCCCGGTGTT.

2.6 Cytokine ELISA

Supernatants of mMSC for cytokine ELISA were generated by plating 5 x 10^4 cells in 24-well plates (BD Falcon) in 1 ml of complete RPMI. After 24 h cells were stimulated with 50 µg/ml pI:C, 5 µg/ml LPS or 500 IU/ml IFNγ. Supernatants were collected after 24 and 72 h. ELISA for interleukin 2 (IL-2), IL-4, IL-6, IL-10, IL-12p40, IL-17A, IFN-γ, and tumor necrosis factor (TNF) (ebioscience) were performed according to manufacturer’s instructions.

2.7 Annexin-PI flow cytometry analysis

For Annexin- and propidium iodide (PI) staining cells were seeded in 6-well plates at a concentration of 10^5/well in complete RPMI. After 24 h cells were treated with 50 µg/ml pI:C or 5 µg/ml LPS for 24 h. Cells were detached using accutase, washed twice with PBS and stained with Annexin V-FITC (BD Pharmingen) and PI (Sigma) for 15 minutes. Flow cytometry was performed using a Cyan ADP Analyzer (DAKO Cytomation) and DAKO Summit software.

2.8 HPLC analysis of tryptophan metabolites

Supernatants of mMSC were obtained as described above. HPLC analysis was performed according to Herve et al. (28) using a Beckman HPLC with PDA detection and Lichrosorb RP-18 column (250mm x 4mm ID, 5µm). Kyn release and tryptophan degradation were measured in complete RPMI 1640 media (Cambrex). The medium was harvested from 24 well plates at the indicated time points, centrifuged and frozen until further analysis. After thawing, the samples were supplemented with trichloroacetic acid for protein precipitation, centrifuged and 100 µl of the supernatant was analyzed by HPLC. Standard curves were generated with L-kyn and L-trp (Sigma) in the same medium. Since FCS contains kyn, low kyn concentrations (~1 µM) were detected in all samples and medium without cells was always measured for comparison.

2.9 Induction and treatment of EAE

Female C57BL/6 mice were immunized subcutaneously (s.c.) with 100 µg/mouse MOG p35-55 in incomplete Freund's Adjuvans (Difco, Detroit, MI, USA) containing 200 µg mycobacterium tuberculosis (Difco) *per* mouse. Concomittantly 200 ng/mouse pertussis toxin diluted in 100 µl of PBS was injected i.v., which was repeated two days later. Clinical signs of disease were scored daily according to a standard scoring system, representing 0 = no clinical signs, 1 = loss of tail tone, 2 = hind limb weakness, 3 = complete hind limb paralysis, 4 = hind limb and forelimb paralysis, 5 = moribund or dead. On day 4 and day 9 after immunization mice were injected intraperitoneally (i.p.) with 10^6 mMSC from C57BL/6 or IDO1-/- mice, diluted in 200 µl of PBS. Control mice received equal volumes of PBS without cells.

2.10 Statistical analyses

Results were assessed by applying Student's t-test statistics to the experimental data obtained *in vitro*. *In vivo* data were statistically evaluated using the Mann-Whitney-U test.

3. Results

3.1 Characterization of mMSC

MSC were isolated from wild-type C57BL/6, IDO-/-, C57BL/6 and SJL/J mice. Flow cytometry demonstrated that the cells were CD9+, CD29+, CD44+, CD90+, CD11b-, CD34-, CD45-, which is indicative of an MSC phenotype (Fig. 1*A*). Staining of calcium deposits verified the capacity to differentiate along the osteogenic lineage (Fig. 1*B*).

Figure 1

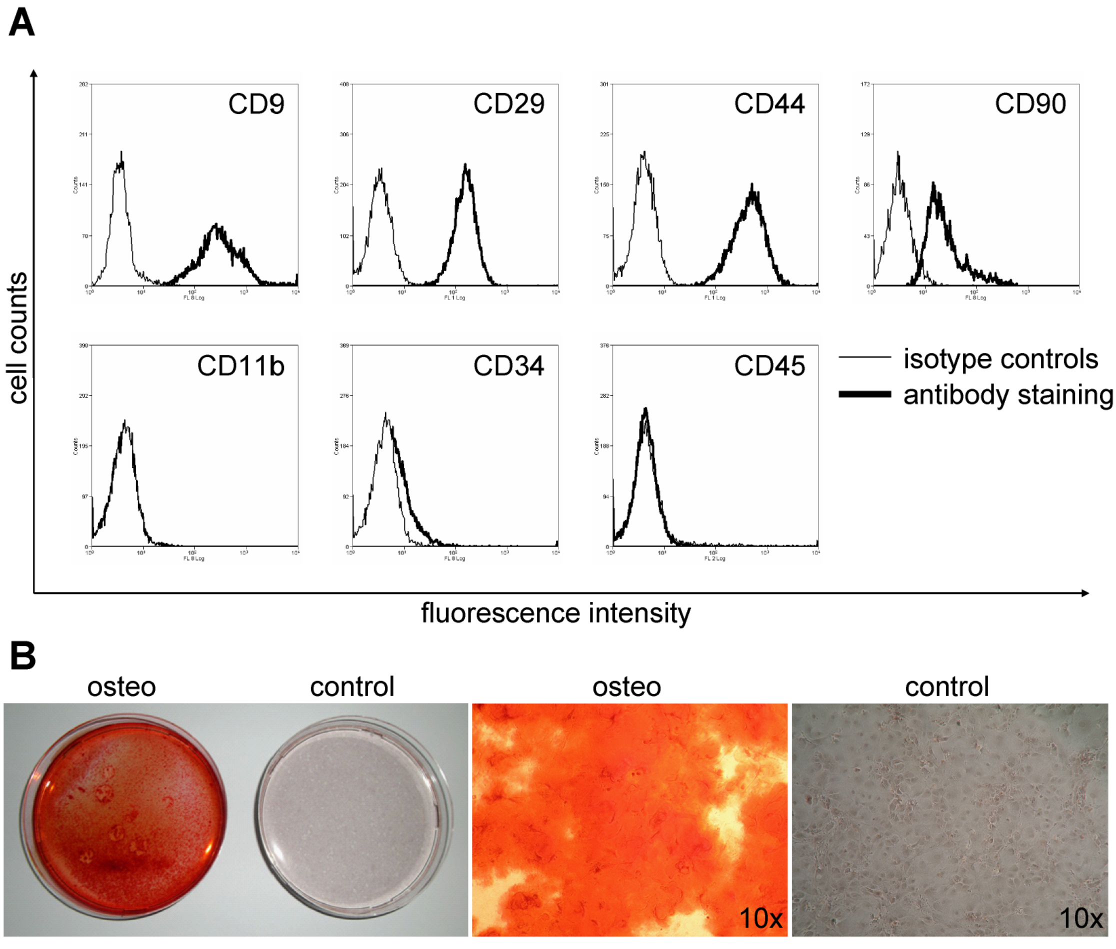

Figure 1. **Characterization of mMSC.** (A) Flow cytometric analysis of the MSC-determining surface markers CD9, CD29, CD44, CD90, CD11b, CD34 and CD45 on mMSC derived from C57BL/6 bone marrow. (B) Osteogenically

differentiated C57BL/6 MSC stained for calcium deposits with Alizarin Red S. Left panel: macroscopic aspect of osteogenically differentiated cells and undifferentiated control cells. Middle and right panel: 10x magnifications. Flow cytometric analyses are representative for at least two independent measurements.

3.2 Tryptophan catabolism in mMSC

Trp catabolism was analyzed in mMSC. To this end mMSC were stimulated with IFN-γ or the TLR ligands pI:C or LPS. Then, mRNA expression of IDO1, the main inducible trp catabolizing enzyme, and the enzymes in its downstream pathway, kynureninase (KYNA), kynurenine monooxygenase (KMO) and 3-hydroxy anthranilic acid oxidase (3-HAAO) (Fig. 2) was determined. Naïve mouse DC served as a positive control. As opposed to mDC, there was no constitutive IDO1 mRNA expression in mMSC. While IFN-γ induced a strong upregulation in both mMSC and mDC, induction of IDO1 mRNA by TLR ligands was weak in mMSC (Fig. 3*A*).

HPLC analysis of trp demonstrated that the increase of IDO1 mRNA in mMSC by TLR ligation or IFN-γ stimulation did not result in IDO1 enzyme activity despite induction of IDO1 mRNA (Fig. 3*B*). Interestingly, kyn in the cell culture media decreased after TLR ligation (Fig. 3*C*), possibly indicating immediate catabolization via the downstream enzymes. We thus analyzed the expression of downstream kyn-catabolizing enzymes. KYNA was constitutively expressed in mDC and – albeit at lower levels – mMSC, but unlike in mDC it was not downregulated in mMSC by TLR ligands or IFN-γ (Fig. 3*D*). IFN-γ or TLR stimulation resulted in downregulation of mRNA expression of KMO and 3-HAAO only in mDC, while mRNA of those two enzymes was not detected in mMSC (Fig. 3*E,F*). Interestingly, when mMSC were stimulated with pI:C or LPS, HPLC analysis detected an additional peak which does not appear after stimulation with IFN-γ but can be increased by combination of TLR ligand and IFN-γ. This additional peak correlated reciprocally with the peak size of kyn, suggesting it may be a derivative of it (Fig. 3*G*). This peak is not identical with trp and all its tested metabolites: kynurenic acid, 3-hydroxykynurenine, anthranilic acid, 3-

hydroxyanthranilic acid, picolinic acid, quinolinic acid, kynuramic acid or nicotinic acid.

Figure 2

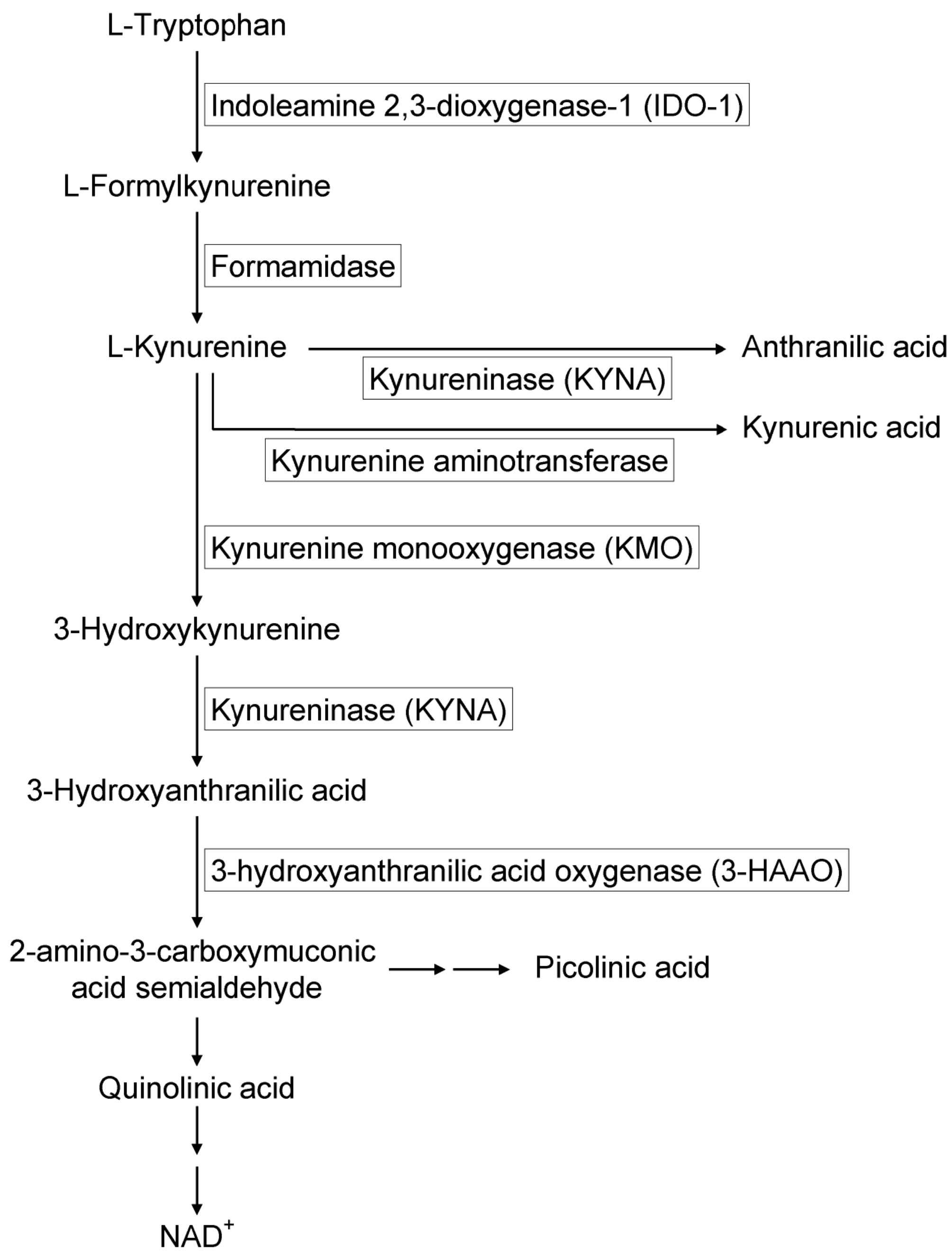

Figure 2. **Catabolic pathway of tryptophan.** (modified from (24))

Figure 3

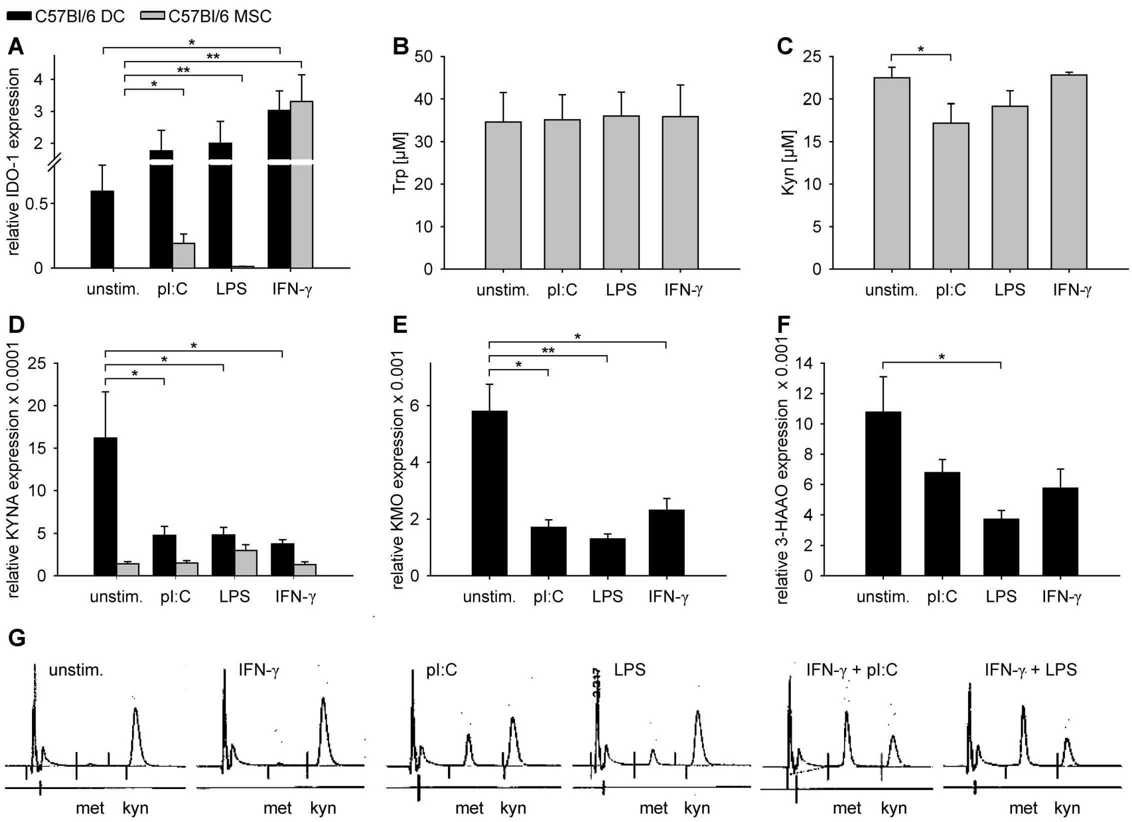

Figure 3. **Effects of TLR ligands and IFN-γ on tryptophan catabolizing enzymes.** (A) Relative expression of IDO1 mRNA in C57BL/6 DC and C57BL/6 MSC after stimulation with TLR ligands or IFN-γ, measured by qRT-PCR. Values are given as transcript abundance relatively to β-actin expression. (B, C) Concentration of trp (B) and kyn (C) in the cell culture supernatant of C57BL/6 MSC after stimulation with TLR ligands or IFN-γ, measured by HPLC. (D) Relative expression of KYNA mRNA in C57BL/6 DC and C57BL/6 MSC after stimulation with TLR ligands or IFN-γ, measured by qRT-PCR. Values are given as transcript abundance relatively to β-actin expression. (E, F) Relative expression of KMO (E) and 3-HAAO (F) mRNA in C57BL/6 DC after stimulation with TLR ligands or IFN-γ, measured by qRT-PCR. Values are given as transcript abundance relatively to β-actin expression. (G) HPLC analysis of kyn and the unknown trp metabolite (met) in the cell culture media of C57BL/6 MSC after stimulation with IFN-γ or TLR ligands as well as in combination. qRT-PCR data

represent mean + SEM of at least two independent experiments, each in duplicates and three serial dilutions. HPLC data are mean ± SEM of at least four independent experiments. * $p < 0.05$ (Student's t-test).

3.3 mMSC express functional TLR

The reduced responsiveness of mMSC to TLR activation is in sharp contrast to hMSC, which display strong IDO1 activity in response to TLR ligation (17). To see whether this reduced responsiveness reflects impaired functionality of TLR in mMSC, we first analyzed TLR mRNA expression. Fig. 4*A* shows that both mDC and mMSC, isolated from C57BL/6 mice, express TLR1-9. Expression levels of mDC and mMSC were comparable except for TLR7, TLR8 and TLR9, which were expressed at orders of magnitude lower in mMSC compared to mDC. TLR3 and 4, the receptors for pI:C and LPS, respectively, were highly expressed in mMSC. mMSC from SJL/J mice showed similar flow cytometric profile and similar TLR expression patterns as C57BL/6 mMSC (Fig. 4*B, C*). To analyze the functionality of TLR3 and TLR4 in mMSC we determined the mRNA expression and release of various cytokines in response to activation with pI:C and LPS. Both pI:C and to a lesser extent LPS induced the release of IL-6 in mMSC and mDC (Fig. 4*D,E*), indicating that the lack of relevant induction of trp catabolism by TLR ligands in mMSC is not due to lack of functional TLR signaling. There was no change in the production of the other tested cytokines: IL-2, IL-4, IL-10, IL-12, IL-17A, IL-23, IFN-γ, and TNF (Table 1). Of note, TLR activation did not result in mMSC death (Fig. 4*F*).

Figure 4

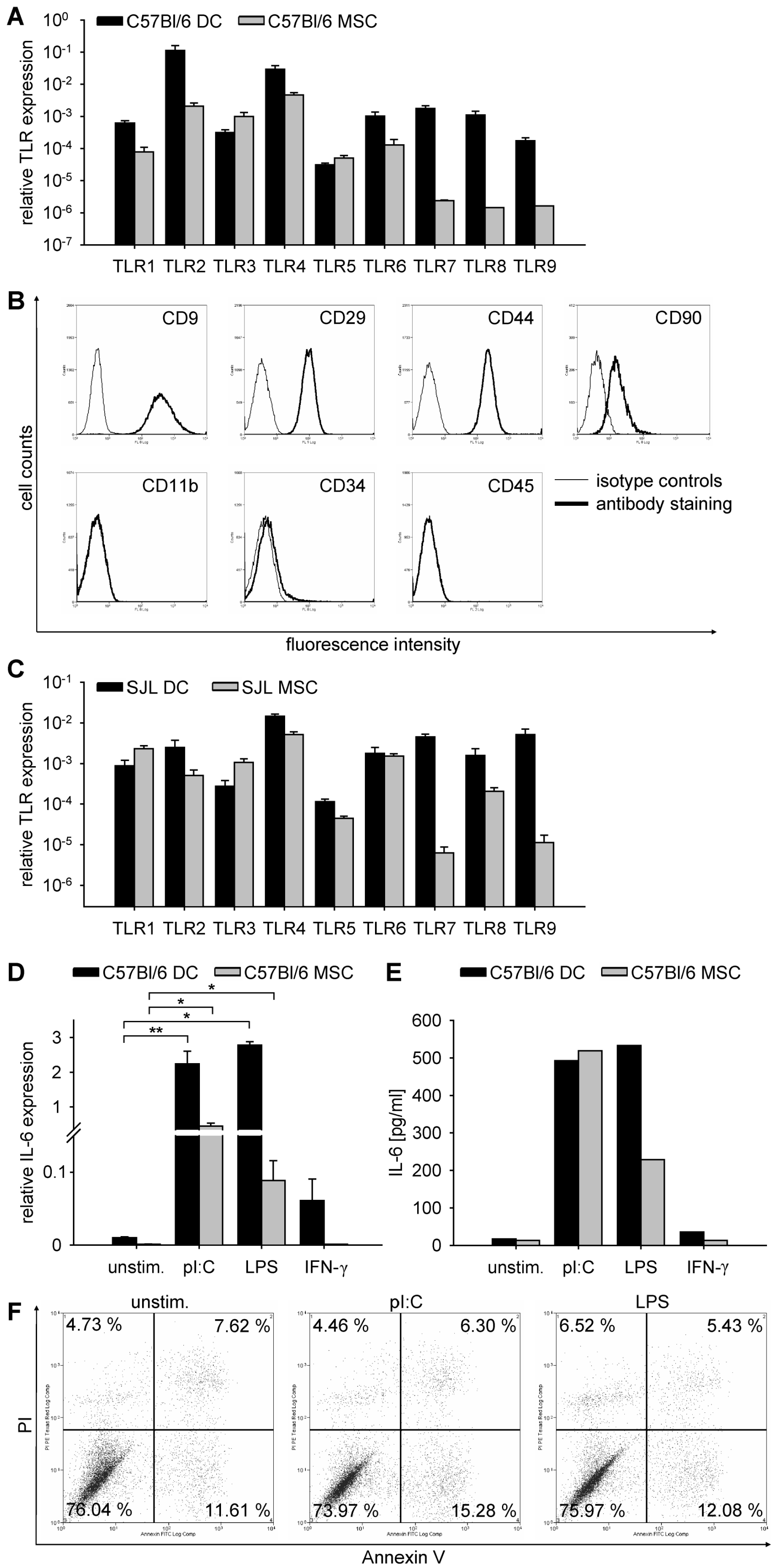
A
C57Bl/6 DC
C57Bl/6 MSC
relative TLR expression
TLR1 TLR2 TLR3 TLR4 TLR5 TLR6 TLR7 TLR8 TLR9
B
CD9
CD29
CD44
CD90
CD11b
CD34
CD45
isotype controls
antibody staining
cell counts
fluorescence intensity
C
SJL DC
SJL MSC
relative TLR expression
TLR1 TLR2 TLR3 TLR4 TLR5 TLR6 TLR7 TLR8 TLR9
D
C57Bl/6 DC
C57Bl/6 MSC
relative IL-6 expression
unstim. pI:C LPS IFN-γ
E
C57Bl/6 DC
C57Bl/6 MSC
IL-6 [pg/ml]
unstim. pI:C LPS IFN-γ
F
unstim.
pI:C
LPS
4.73 %
7.62 %
76.04 %
11.61 %
4.46 %
6.30 %
73.97 %
15.28 %
6.52 %
5.43 %
75.97 %
12.08 %
PI
Annexin V

Figure 4. **TLR expression on mMSC.** (A) Relative TLR 1-9 mRNA expression in C57BL/6 DC or C57BL/6 MSC, measured by qRT-PCR. Values are given as transcript abundance relatively to β-actin expression. (B) Flow cytometric analysis of MSC-determining surface markers on MSC derived from bone marrow of SJL/J mice. (C) Relative TLR 1-9 mRNA expression in SJL/J DC and SJL/J MSC, measured by qRT-PCR. Values are given as transcript abundance relatively to β-actin expression. (D) Relative IL-6 mRNA expression in C57BL/6 DC and C57BL/6 MSC after stimulation with TLR ligands or IFN-γ, measured by qRT-PCR. Values are given as transcript abundance relatively to β-actin expression. (E) IL-6 production, measured by ELISA in supernatants of C57BL/6 DC and C57BL/6 MSC after stimulation with TLR ligands or IFN-γ. (F) C57BL/6 MSC cell death analysis by FACS analysis of Annexin V and PI after stimulation with TLR ligands. qRT-PCR data are mean + SEM from 3 independent experiments, each performed in duplicates and three serial dilutions. * $p < 0.05$; ** $p < 0.005$ (Student's t-test). Flow cytometric analyses are representative data for at least two independent measurements. ELISA data are representative values from one of two independent experiments.

Table 1

	unstimulated			**pI:C**			**LPS**			**IFN-γ**		
	wt DC	**wt MSC**	**-/- MSC**	**wt DC**	**wt MSC**	**-/- MSC**	**wt DC**	**wt MSC**	**-/- MSC**	**wt DC**	**wt MSC**	**-/- MSC**
IL-2	*nd*	*nd*	*nd*	*nd*	*nd*	*nd*	*nd*	*nd*	*nd*	*nd*	*nd*	*nd*
IL-4	*nd*	*nd*	*nd*	*nd*	*nd*	*nd*	*nd*	*nd*	*nd*	*nd*	*nd*	*nd*
IL-6	27	9	1	823	566	455	839	355	128	40	1	2
IL-10	52	41	53	41	83	101	52	65	73	59	47	50
IL-12	7	10	18	73	12	23	210	14	17	3	13	34
IL-17	*nd*	*nd*	*nd*	*nd*	*nd*	*nd*	*nd*	*nd*	*nd*	*nd*	*nd*	*nd*
IFN-γ	3	1	1	9	14	20	1	3	1	*n/a*	*n/a*	*n/a*
TNF	11	9	11	24	19	8	16	15	11	10	10	13

Table 1. **IDO1-/- and wt mMSC express functional TLR.** Production of IL-2, IL-4, IL-6, IL-10, IL12/23p40, IL-17, IFN-γ and TNF, measured by ELISA in cultured DC, and IDO-/- MSC or wt MSC. Values are given as mean pg/ml from duplicates. Data are representative of 2 independent experiments. nd: no cytokine detection, n/a: not applicable.

3.4 mMSC suppress myelin-specific T cells

As a *in vitro* model for antigen-specific T cell immunity we chose myelin-specific T cells from 2D2 mice with a transgenic TCR specific for the immunodominant peptide p35-55 of MOG expressed on CD4+ T cells. MOG p35-55 specific IFN-γ-secreting TH1-polarized T cells can be induced to become encephalitogenic in EAE (26). IFN-γ, in turn, induces IDO1 expression in mMSC (Fig. 3*A*). To evalute whether mMSC suppress the proliferation of MOG-specific CD4+ T cells, splenocytes from 2D2 mice were activated with MOG p35-55 in the presence of syngeneic mMSC. Fig. 5*A* shows that mMSC suppress the proliferation of MOG-specific CD4+ T cells in a concentration-dependent manner. Adding 1-L-MT, a competitive IDO1 inhibitor (17), to the mMSC/splenocyte culture did not restrict the capacity of mMSC to suppress T cell proliferation, indicating that mMSC-derived IDO1 is not involved in the suppression of T cell proliferation (Fig. 5*B*). Of note, in the absence of mMSC, 1-L-MT led to a 25 % reduction of T cell proliferation indicating that 1-L-MT may interfere with our system independently of IDO1 activity. Moreover, pretreatment of mMSC with IFN-γ to induce IDO1 did not increase the immunosuppressive capacities of mMSC (Fig. 5*C*). Also, stimulation of mMSC with pI:C and LPS did not have any effect on T cell proliferation (Fig. 5*D*).

Figure 5

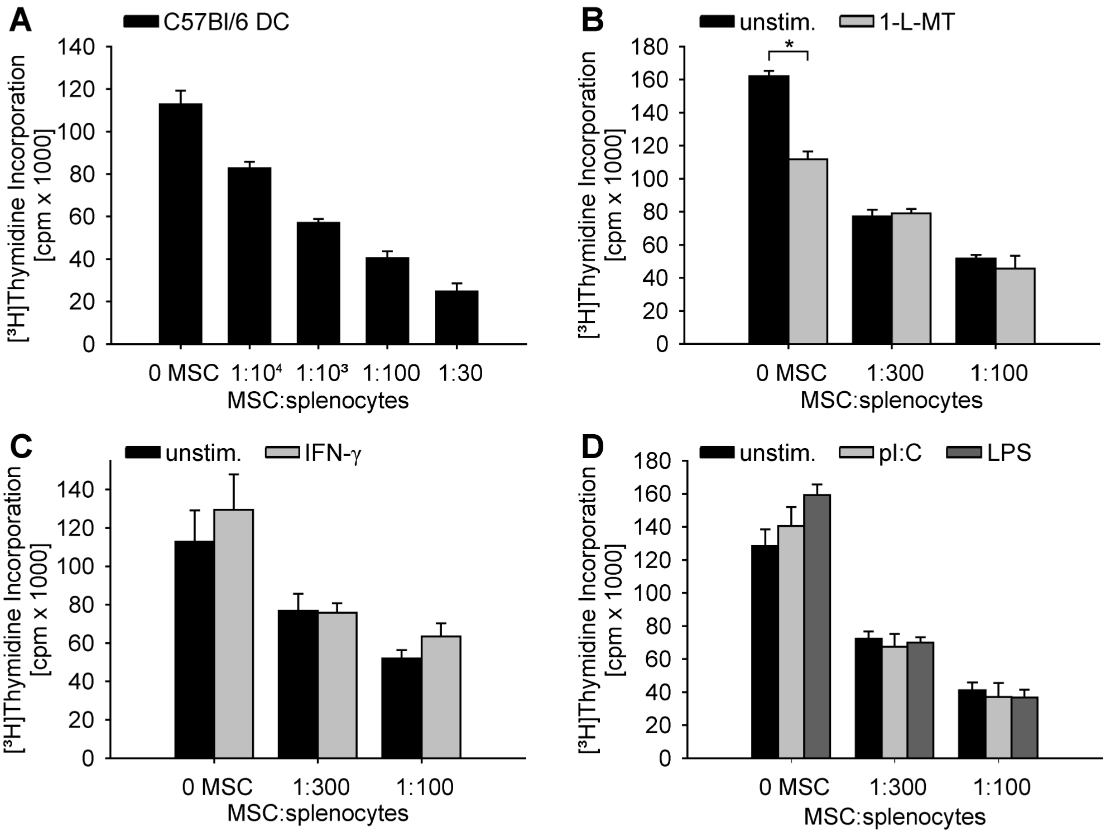

Figure 5. **IDO1-independent immunosuppression by mMSCs.** (A-D) Proliferation of MOG p35-55 activated 2D2 splenocytes in coculture with C57BL/6 MSC at various ratios, quantified by ^{3}H-thymidine incorporation. (B) 1-L-MT or vehicle control was added during the stimulation with peptide. (C,D) mMSC were preactivated with IFN-γ (C), pI:C or LPS (D). Data in (A) show mean + SEM of triplicates of one representative experiment out of 10, data in (B-D) represent mean + SEM of three independent T cell proliferation assays. All experiments were carried out in triplicates and measured by ^{3}H-thymidine uptake. * $p < 0.05$ (Student's t-test).

3.5 IDO1 is not involved in mMSC-mediated immunosuppression in vitro

To further substantiate the finding that IDO1 is not involved in mMSC-mediated inhibtion of MOG-specific T cells, we used mMSC from IDO1-deficient mice. IDO1-/- mMSC did not differ substantially from wild-type C57BL6 mMSC with respect to phenotype, differentiation capacity, TLR expression and IL-6 induction (Fig. 6*A*-*D*). When incubated with 2D2 splenocytes activated with MOG p35-55, IDO1-/- mMSC displayed similar immunosuppressive activity as their wildtype counterparts, indicating that IDO1 is not involved in mMSC-mediated modulation of TH cell activation (Fig. 6*E*).

Figure 6

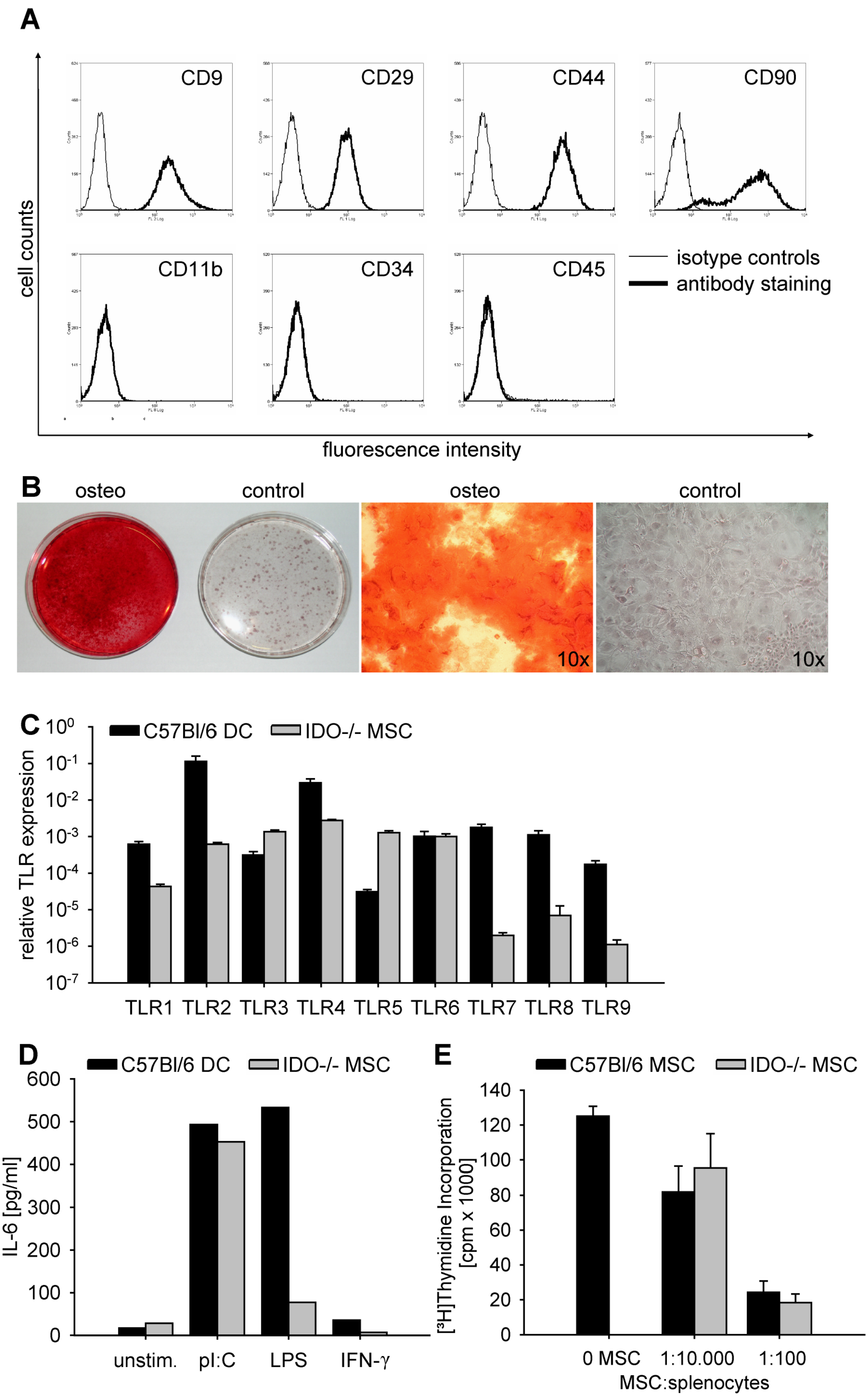
A
CD9
CD29
CD44
CD90
CD11b
CD34
CD45
cell counts
isotype controls
antibody staining
fluorescence intensity
B
osteo
control
osteo
control
10x
10x
C
C57Bl/6 DC
IDO-/- MSC
relative TLR expression
10^0
10^-1
10^-2
10^-3
10^-4
10^-5
10^-6
10^-7
TLR1 TLR2 TLR3 TLR4 TLR5 TLR6 TLR7 TLR8 TLR9
D
C57Bl/6 DC
IDO-/- MSC
IL-6 [pg/ml]
600
500
400
300
200
100
0
unstim.
pI:C
LPS
IFN-γ
E
C57Bl/6 MSC
IDO-/- MSC
[³H]Thymidine Incorporation [cpm x 1000]
140
120
100
80
60
40
20
0
0 MSC
1:10.000
1:100
MSC:splenocytes

Figure 6. **IDO1 is dispensable for mMSC mediated immunosuppression *in vitro*.** (A) Flow cytometry analysis of MSC-determining surface markers on MSC derived from bone marrow of IDO1-/- mice. (B) Osteogenically differentiated IDO-/- mMSC stained for calcium deposits with Alizarin Red S. Left panel: macroscopic aspect of osteogenically differentiated cells and undifferentiated control cells. Middle and right panel: 10x magnifications. (C) Relative TLR 1-9 mRNA expression in C57BL/6 DC and IDO-/- MSC, measured by qRT-PCR. Values are given as transcript abundance relatively to β-actin expression. (D) IL-6 production, measured by ELISA in supernatants from C57BL/6 DC and IDO1-/- MSC after stimulation with TLR ligands or IFN-γ. (E) Proliferation of 2D2 splenocytes in coculture with wildtype C57BL/6 MSC or IDO1-/- MSC in different ratios, quantified by ^{3}H-thymidine incorporation. Flow cytometry analyses are representative for two independent measurements. qRT-PCR data are mean + SEM from three independent experiments, each performed in duplicates and three serial dilutions respectively. ELISA data are representative values from one of two independent experiments. Proliferation data represent mean + SEM of three independent T cell prolifation assays, each carried out in triplicates and measured by ^{3}H-thymidine uptake.

3.6 IDO1 is not involved in mMSC-mediated immunosuppression in EAE

We next analyzed the role of mMSC trp catabolism *in vivo* by immunizing C57BL/6 mice with MOG p35-55 to induce EAE. Systemic administration of mMSC led to an attenuation of disease severity as determined by the grade of paralysis. IDO-/- mMSC were equally effective in reducing disease severity as wildtype mMSC (Fig. 7*A*). We then used lymph node cells of immunized animals of all three groups and restimulated them *in vitro* with MOG p35-55. Lymphocytes from animals treated with wildtype or IDO1-/- mMSC showed reduced proliferation compared to lymphocytes from the control group. There was no difference between wildtype and IDO1-/- mMSC treated groups (Fig. 7B). While the production of the T cell cytokines IL-2, IL-4, IL-6, IL-10 and IL-12/23p40 remained unchanged, treatment with both wildtype and IDO1-/- mMSC led to an equal reduction of IL-17 release from lymph node cells after restimulation with

MOG p35-55 *ex vivo*. Interestingly TNF and IFN-γ seemed to be even more suppressed by IDO-/- mMSC than by the wildtype mMSC (Fig. 7*C*).

Figure 7

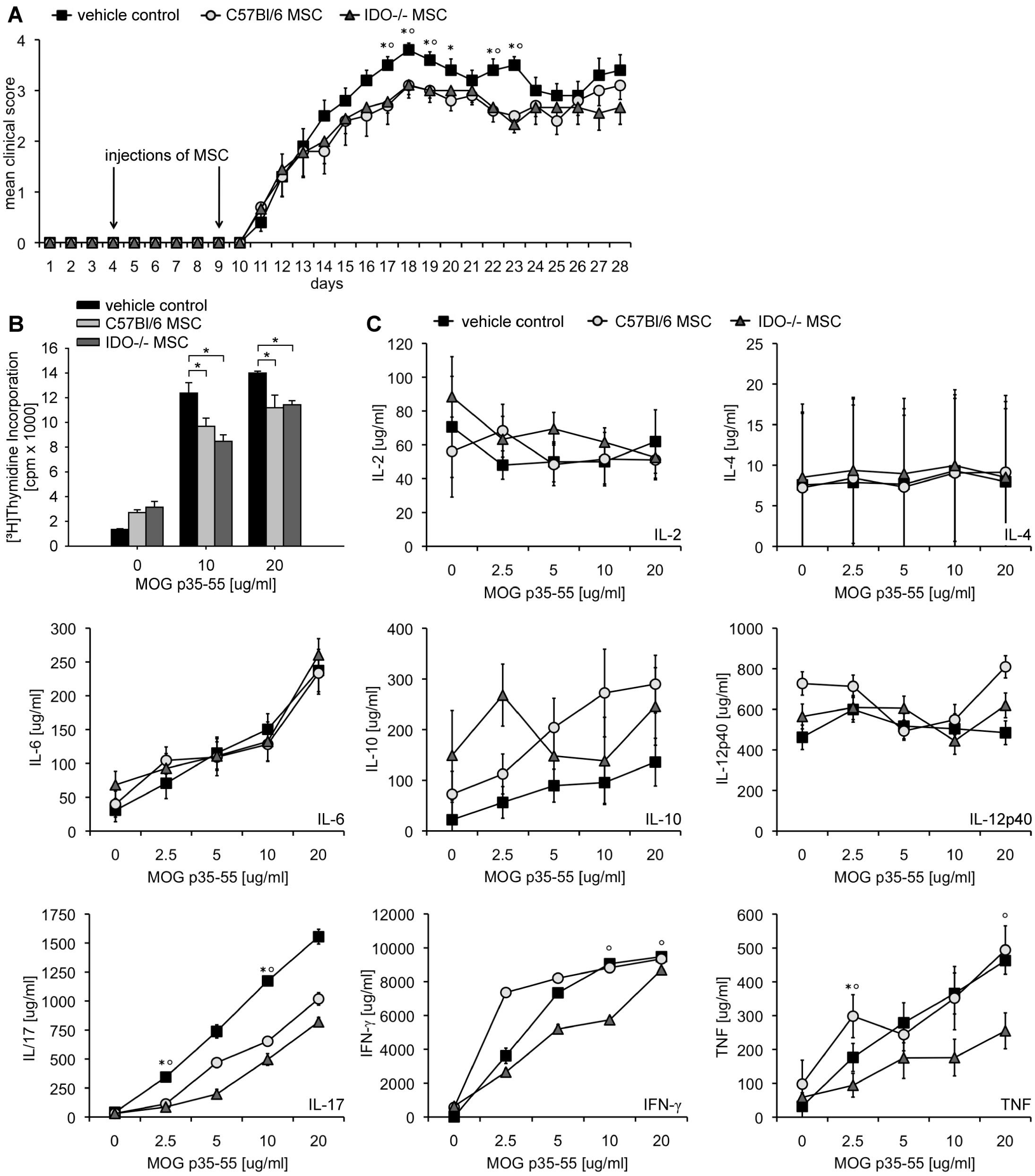

Figure 7. **IDO1 is dispensable for mMSC mediated immunosuppression *in vivo*.** (A) EAE disease scores, followed-up for 28 days. Animals were treated with two i.p. injections of vehicle control, wildtype C57BL/6 MSC or IDO-/- MSC on day 4 and day 9 after immunization, means ± SEM are shown, n = 10 mice per group, * $p < 0.05$ between wildtype MSC treated group and controls, ° $p < 0.05$ between IDO-/- MSC treated group and controls according to Mann-Whitney-U-test. (B) Proliferation of MOG p35-55-restimulated lymphocytes from EAE mice treated with vehicle control, wildtype C57BL/6 MSC or IDO1-/- MSC. (C) Production of IL-2, IL-4, IL-6, IL-10, IL12/23p40, IL-17, IFN-γ and TNF, measured by ELISA in MOG p35-55 restimulated lymphocyte cultures of mice treated with vehicle control, wildtype C57BL/6 MSC or IDO1-/- MSC. Cells were pooled from 10 mice per group and assays were carried out in triplicates, means ± SEM are shown, * $p < 0,05$ between wildtype C57BL/6 MSC treated group and controls, ° $p < 0,05$ between IDO-/- MSC treated group and controls (Student's t-test).

4. Discussion

MSC have the unique ability to suppress multiple cellular components of the immune system. The clinical development of hMSC as a source for tissue regeneration and as vehicles to deliver immunosuppressive signals to inflamed tissues after systemic administration has made rapid progress in the past several years. A recent phase II clinical trial has shown efficacy of systemic treatment of patients with steroid-resistant acute graft-versus-host disease with allogeneic bone marrow-derived hMSC (29). Potential clinical applications have been extended to autoimmune diseases such as MS and rheumatoid arthritis, where preclinical mouse models have demonstrated the efficacy of autologous bone marrow-derived MSC (15). The mechanism of immunosuppressive action of MSC has not been fully elucidated but appears to involve membrane bound and soluble factors. Among the soluble factors, the oxidative catabolism of trp along the kyn pathway is an important immunosuppressive mechanism restricting antigen-specific T cell responses in autoimmunity (23). This pathway is operative in hMSC and delivers immunosuppressive signals to T cells. We have recently shown that TLR ligands activate immunosuppressive trp catabolism via IDO1 in hMSC and that inhibition of IDO1 reverses hMSC-mediated T cell paralysis (17). This study was initiated to examine the contribution of IDO1-mediated immunosuppression to the therapeutic efficacy of mMSC in a mouse model of MS.

Using a transgenic mouse model with CD4+ T cells carrying a TCR specific for an immunodominant epitope of MOG we show that autologous bone-marrow derived mMSC suppress proliferation of MOG-specific T cells (Fig. 5A) supporting the notion that suppression of encephalitogenic myelin-specific T cells is involved in the therapeutic efficacy of MSC in EAE (9). We show, however, that as opposed to human MSC, mouse MSC cannot be activated to express functional IDO1 activity by stimulation with TLR ligands or with IFN-γ. This inability to catabolize trp is likely to be species- and not strain-dependent since neither C57BL/6 MSC (Fig. 3B) nor phenotypically similar SJL/J MSC (Fig. 4B,C) displayed IDO1 enzyme activity (data not shown). This finding is in line with in-vitro data from previous studies that failed to detect IFN-γ-inducible IDO1 enzyme activity in murine MSC cell lines (30, 31). Interestingly, IFN-γ induced a strong IDO1 mRNA expression comparable to DC (Fig. 2A), pointing to posttranscriptional or posttranslational mechanisms regulating IDO1 enzyme activity. An identical discrepancy between IDO1 mRNA expression and enzyme activity has been observed in CD8α+ DC (32). The mechanisms involved in posttranscriptional and posttranslational regulation of IDO1 enzyme activity remain elusive but may involve nitration of the enzyme by peroxynitrite (33).

As we and others measured IDO1 activity by determining the concentration of kyn in the cell culture supernatant via HPLC it was conceivable that kyn produced from trp after IFN-γ stimulation is catabolized further to downstream metabolites such as 3-hydroxy-kynurenine and 3-hydroxy-anthranilic acid. The fact that trp levels in the supernatant of IFN-γ-stimulated mMSC did not decrease and that the kyn-catabolizing enzymes are downregulated rather than upregulated renders this hypothesis unlikely. Interestingly, stimulation with TLR ligands resulted in catabolism of kyn present in the cell culture media as evidenced by a reduction of kyn and simultaneous accumulation of a kyn catabolite in the supernatant (Fig. 3C,G). The kyn catabolite is unlikely due to activity of the kynurenine catabolising enzyme KYNA as this enzyme is suppressed rather than induced by pI:C or LPS. KMO and 3-HAAO are not expressed by mMSC (Fig. 3D-F). Moreover the unidentified kyn catabolite (Fig. 3G) is not one of the classic catabolites such as kynurenic acid, 3-hydroxykynurenine, anthranilic acid, 3-hydroxyanthranilic acid, picolinic acid, quinolinic acid, kynuramic acid or nicotinic acid as evidenced by HPLC analysis (data not shown). We are currently investigating the identity of this kyn catabolite.

The failure of the TLR ligands pI:C and LPS to induce functional trp catabolism led us to investigate whether TLR signaling in general is operational in bone marrow-derived mMSC. We show that mMSC express TLR1-9 mRNA at levels comparable to mDC, except for TLR7, TLR8 and TLR9 whose expression levels are three orders of magnitude lower than in mDC (Fig. 4A,C, 6C). These results are in line with another study in mMSC (34). As in human MSC (17) TLR3 and TLR4 are expressed at levels comparable to DC (Fig. 4A,C, 6C). We show that the TLR pathway is not dysfunctional *per se* (Fig. 4D,E, 6D) indicating that - unlike in hMSC - activation of the TLR pathway alone is not sufficient to activate functional IDO1 in mMSC. These results identify an important immunological difference between human and murine MSC. In this respect mMSC are similar to hDC where TLR activation requires a second signal to induce IDO1 (35). It is tempting to speculate that the strong induction of an autocrine IFN-β loop by TLR ligation in hMSC (17) which is shared by plasmacytoid DC (36) is the critical determinant of the discrepancy between mMSC and hMSC.

Since these results did not rule out that IDO1 could be induced by other signals delivered through interacting T cells or myeloid cells we tested whether inhibition of IDO1 would lead to an incapacitation of mMSC to suppress the activation of myelin-specific T cells. Treatment with the competitive IDO1 inhibitor 1-L-MT did not rescue the immunosuppressive phenotype in this setting (Fig. 5B). Interestingly, in the absence of mMSCs 1-L-MT suppressed T cell proliferation not only indicating that IDO1-mediated trp catabolism does not restrict T cell proliferation in this system but also suggesting that 1-L-MT may have off-target effects. It has been reported that 1-MT modulates the function of APC independently of IDO1 inhibition (37). To more specifically delineate the role of IDO1 in immunosuppression of mMSC we generated MSC from bone marrow cells of IDO1-deficient mice. IDO1-/- MSC were phenotypically similar to wildtype MSC with respect to surface marker expression, differentiation, expression of TLR and IL-6 induction (Fig. 6A-D). IDO1 deficiency in mMSC neither reversed mMSC-mediated suppression of antigen-specific T cell proliferation *in vitro* (Fig. 6E) nor did it alter the therapeutic effects of mMSC in a mouse model of MS (Fig. 7A). Interestingly, both wildtype and IDO1-/- MSC led to a reduction of IL-17 production in myelin-specific CD4+ T cells *ex vivo* while IFN-γ and TNF release was even more suppressed by IDO-deficient mMSC (Fig. 7C). Rafei and colleagues have recently demonstrated a suppression of both, TH1 and TH17-immunity by MSC (8, 38). The indistinguishable clinical outcome and the lack of differences in TH cell proliferation between the two groups may indicate that mMSC are responsible for TH17 suppression rather than TH1 polarization of myelin-specific T cells *in vivo*. TH17 cells have shown to be critically involved in the pathogenesis of EAE, although their ultimate importance in EAE and in other models of autoimmune diseases remains controversial (39).

5. Conclusion

We show that IDO1 is dispensable for the immunosuppressive signals delivered by mMSC and for the therapeutic effects of autologous mMSC in autoimmune neuroinflammation. This is in sharp contrast to human MSC where IDO1 is critically involved in the suppression of T cell immunity (16, 17). The preclinical development of MSC as a cell-based therapy for autoimmune diseases in mouse models using autologous bone marrow-derived MSC should therefore be interpreted with caution with respect to its prediction for the therapeutic efficacy of hMSC in human autoimmune diseases. Using hMSC in mouse models, which can effectively be done without obvious evidence of immune rejection (40) may be more appropriate for preclinical trials characterizing and optimizing route of administration, motility, cellular targets and immunosuppressive mechanisms of MSC in disease settings.

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7. Author's Contributions

Tobias V. Lanz: designed research, performed all experiments, wrote the paper and the dissertation

Christiane A. Opitz: helped with experiments, discussed the topic

Peggy P. Ho: helped with the animal experiments

Ankur Agrawal: helped with the cytokine ELISA experiments

Christian Lutz: helped with the HPLC measurements

Michael Weller: provided lab space and funding, discussed and revised the paper

Andrew L. Mellor: provided IDO1-/- mice

Lawrence Steinman: provided lab space and facilities for animal experiments, discussed and revised the paper

Wolfgang Wick: provided lab space and funding, discussed and revised the paper

Michael Platten: designed research, provided lab space and funding, discussed the topic, instructed TVL in the experimental methods, revised the paper

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Lebenslauf

Persönliche informationen

Geburtsdatum: 3.1.1983

Geburtsort: Mainz, Deutschland

Nationalität: deutsch

Ausbildung

1989 – 1993 Gaisental-Grundschule Biberach

1993 – 2002 Wieland-Gymnasium Biberach

18.6.2002 Abitur

08/2002 – 09/2002 und 10/2003 Ausbildung zum Rettungshelfer und Rettungssanitäter

10/2002 – 05/2003 Zivildienst als Rettungshelfer beim Roten Kreuz Biberach

Seit 03/2003 Medizinstudium an der Eberhard-Karls-Universität Tübingen

17.3.2005 Physikum

02/2009 – 01/2010 praktisches Jahr an der Universitätsklinik Tübingen und am Hospital for Neurology and Neurosurgery, Queen Square, London

18.6.2010 Staatsexamen

Klinische Erfahrung

09/2002 Praktikum in der Anästhesie, Kreiskrankenhaus Biberach

09/2002 – 05/2003 Arbeit als Rettungssanitäter

09/2003 Praktikum in der Anästhesie, Kreiskrankenhaus Biberach

03 – 04/2004 Pflegepraktikum auf der Intensivstation, Kreiskrankenhaus Biberach

08/2005 Famulatur in der Neurologie, Krankenhaus Bogenhausen, München

10/2005 Famulatur in der Kardiologie, Universitätsklinik Tübingen

08/2008 Famulatur in der Unfallchirurgie/Orthopädie, Katharinen-Hospital Stuttgart

09/2008 Famulatur in der Dermatologie, Hautklinik der Universität Tübingen

10/2008 Famulatur in der Neuroophthalmologie, Augenklinik der Universität Tübingen

Promotion und Forschung

01/2006 – 10/2007 Durchführung der vorliegenden Promotionsarbeit am Hertie-Institut für klinische Hirnforschung Tübingen

10/2007 – 07/2008: Forschungsaufenthalt an der Stanford-University, Forschungsarbeit im Labor von Prof. Lawrence Steinman

Stipendien und Preise

Stipendium der Studienstiftung des Deutschen Volkes

Stipendium des interdisziplinären Zentrums für klinische Forschung (IZKF) "Molekulare Medizin" der medizinischen Fakultät Tübingen

Stipendium des Biomedical Exchange Programs, BMEP der Medizinischen Universität Hannover

IZKF Poster-Preis 2006 der medizinischen Fakultät Tübingen

Veröffentlichungen

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