Mesenchymal stem cells transfer mitochondria to allogeneic Tregs in an HLA-dependent manner improving their immunosuppressive activity

Karolina Piekarska1,2, Zuzanna Urban-Wójciuk3, Małgorzta Kurkowiak3, Iwona Pelikant-Małecka4,5,6, Adriana Schumacher7, Justyna Sakowska6,2, Jan Henryk Spodniki8, Łukasz Arcimowicz3, Hanna Zielińska2, Bogusław Tymoniuk6,9, Alicja Renkielska10, Janusz Siebert1,11, Ewa Słomińska4, Piotr Trzonkowski2, Ted Hupp3,12 & Natalia Maria Marek-Trzonkowska1,2,3,✉

Cell-based immunotherapies can provide safe and effective treatments for various disorders including autoimmunity, cancer, and excessive proinflammatory events in sepsis or viral infections. However, to achieve this goal there is a need for deeper understanding of mechanisms of the intercellular interactions. Regulatory T cells (Tregs) are a lymphocyte subset that maintain peripheral tolerance, whilst mesenchymal stem cells (MSCs) are multipotent nonhematopoietic progenitor cells. Despite coming from different origins, Tregs and MSCs share immunoregulatory properties that have been tested in clinical trials. Here we demonstrate how direct and indirect contact with allogenic MSCs improves Tregs’ potential for accumulation of immunosuppressive adenosine and suppression of conventional T cell proliferation, making them more potent therapeutic tools. Our results also demonstrate that direct communication between Tregs and MSCs is based on transfer of active mitochondria and fragments of plasma membrane from MSCs to Tregs, an event that is HLA-dependent and associates with HLA-C and HLA-DRB1 eplet mismatch load between Treg and MSC donors.

1Department of Family Medicine, Medical University of Gdańsk, Gdańsk, Poland. 2Department of Medical Immunology, Medical University of Gdańsk, Gdańsk, Poland. 3International Centre for Cancer Vaccine Science, University of Gdańsk, Gdańsk, Poland. 4Department of Biochemistry, Medical University of Gdańsk, Gdańsk, Poland. 5Division of Medical Laboratory Diagnostics, Medical University of Gdańsk, Gdańsk, Poland. 6Biobanking and Biomolecular Resources Research Infrastructure Poland (BBMRI-PL), Gdańsk, Poland. 7Department of Pharmacology, Medical University of Gdańsk, Gdańsk, Poland. 8Department of Anatomy and Neurobiology, Medical University of Gdańsk, Gdańsk, Poland. 9Department of Immunology and Allergy, Medical University of Łódź, Łódź, Poland. 10Department of Plastic Surgery, Medical University of Gdańsk, Gdańsk, Poland. 11University Center for Cardiology, Gdańsk, Poland. 12Cell Signaling Unit, Institute of Genetics and Molecular Medicine, University of Edinburgh, Edinburgh, United Kingdom. ✉email: natalia.marek-trzonkowska@ug.edu.pl
Regulatory T cells (Tregs) are a unique leukocyte subset that does not protect from infectious agents but controls activation of other immune cells. Despite Tregs account for ~1% of peripheral blood lymphocytes, they maintain self-tolerance. Lack of Tregs leads to the development of multiple autoimmune diseases and severe hypersensitivities as it is manifested in immune dysregulation, polyendocrinopathy, enteropathy, X-linked syndrome (IPEX). In addition, Tregs play a crucial role in graft tolerance and survival, while their higher numbers in allogeneic hematopoietic stem cell grafts predict improved survival of the recipient after transplantation. Our group has a long-term experience in clinical use of Tregs. We were the first who administered Tregs in patients with graft versus host disease (GVHD), in type 1 diabetes (DM1) and multiple sclerosis. We and others have also observed that Treg-based therapies are the most beneficial in the early stage of disease and are less effective after excessive immune system activation. In addition, the beneficial effect of a therapy with ex vivo expanded Tregs wanes with time. Therefore, there is a need for strategies that will enhance immunoregulatory potential of these cells. We believe that the proper in vitro Treg conditioning may result in the production of cells that will completely stop progression of the excessive immune responses or prolong clinical improvement significantly more than it was observed before.

Mesenchymal stromal/stem cells (MSCs) are multipotent cells capable of differentiation into multiple cell types of mesenchymal and non-mesenchymal origin, including chondrocytes, osteoblasts, adipocytes, glial cells, neurons, epithelial cells and hepatocytes (e.g. pneumocytes, retinal pigment epithelium and renal tubular epithelial cells). MSCs can be isolated from bone marrow, adipose tissue, umbilical cord, Wharton’s jelly, amniotic fluid, gingiva, tooth pulp, periodontal tissue and in general from connective tissue of most organs. Besides their regenerative properties, MSCs have shown to express low levels of MHC class I molecules and thus to explain why multiple clinical trials have been conducted based on transfer of active mitochondria and fragments of the plasma membrane. These data elucidate mechanisms that underlay cell communication and hopefully will initiate studies on interactions between Tregs and non-immune cells.

**Results**

Treg contact with allogeneic ASCs induces expression of CD69 and preserves CD25High phenotype in Tregs, but only direct cell-to-cell communication prevents loss of FoxP3 during culture in vitro. Just after isolation Tregs were expanded alone for 7 days to obtain the required number of cells for further experimentation. A set of cultures were then launched to assess the consequences of direct and indirect contact of Tregs with allogeneic ASCs. Tregs were expanded alone (monocultures), Tregs were cultured in cell-to-cell contact with allogeneic ASCs (direct cocultures) or Tregs were separated from allogeneic ASCs with a cell impermeant membrane with a pore size of 0.4 μm that prevented passage of the particles larger than 0.4 μm (indirect cocultures). Treg monocultures supplemented with supernatants (SN) derived from ASC monocultures served as a control.

The cells were processed after the next 7 days (14 days after Treg isolation) and the data showed that Tregs cultured in direct cell-to-cell contact with ASCs exhibit the highest frequency of FoxP3+ cells as compared with other culture conditions. The median % of FoxP3+ Tregs in monocultures, in indirect cocultures and in monocultures supplemented with ASC-derived SNs was as follows: 33.7, 85.5 and 84.8%. At the same time in the cocultures where Tregs were expanded in direct contact with ASCs, the FoxP3+ population comprised 90% of the T cells. The difference in % of FoxP3+ cells in this culture condition and standard monoculture was statistically significant (Mann–Whitney U test; MW; p = 0.02; Fig. 1a). However, neither direct nor indirect contact with ASCs affected the intensity of FoxP3 expression by FoxP3+ cells (Supplementary Fig. 1). In addition, direct and indirect contact with allogeneic ASCs resulted in higher frequency of CD25High cells among FoxP3+ Tregs as compared with monocultures (MW; p = 0.04 and p = 0.03, respectively; Fig. 1b). A significant difference between the conditions was observed for the expression of the activation marker CD69. Tregs cocultured in direct and indirect contact with allogeneic ASCs were characterized by significantly higher numbers of CD69+ cells, than those derived from both standard and SN supplemented monocultures (MW; p = 1 × 10^-4 for direct and indirect cocultures vs standard monocultures, p = 4 × 10^-3 for direct and indirect cocultures vs SNs; Fig. 1d). However, the highest frequency of CD69+ Tregs was observed in the direct cocultures (MW; p = 1 × 10^-4 for direct vs indirect cocultures). In addition, direct and indirect contact with allogeneic ASCs increased the intensity of CD69 expression on the surface of CD69+ Tregs (MW; p = 1 × 10^-4 for direct and indirect cocultures vs standard monocultures, p = 4 × 10^-3 for direct and indirect cocultures vs SNs; Supplementary Fig. 1d). No statistically significant differences were found amongst the different Treg
Fig. 1 Direct contact with allogeneic ASCs induces high expression of CD69 and preserves expression of FoxP3 and CD25 in Tregs during culture in vitro. Graphs and representative dot-plots depict data for Tregs from monocultures at day 7 and day 14 after isolation (Solo day 7 and Solo day 14, respectively), from direct and indirect cocultures at day 14 after Treg isolation that corresponds with day 7 of coculture (Direct day 14 and Indirect day 14, respectively), and from Treg monocultures supplemented with ASC-derived SNs at day 14 after isolation (corresponds with day 7 of culture supplementation with SNs; SN day 14). 

(a) The frequency of FoxP3+ cells within CD4+ T cell population.

(b) The frequency of CD25High, c Helios+ and d CD69+ cells within the CD4+ FoxP3+ Treg subset.

* p < 0.05 for comparisons between the given condition vs Solo day 14. ** p < 0.05 for differences between Direct day 14 vs Indirect day 14. *** p < 0.05 for differences between the given condition vs SN day 14. 

e) Gating strategy for identification of Tn, Tcm and Tem Tregs. f) Proportions of naive and memory subsets in the indicated culture conditions. * p < 0.05 for comparisons of Tn, Tcm and Tem subset frequencies between day 7 and 14 in the indicated culture conditions. ** p < 0.05 for comparisons of Tem subset frequencies in the indicated culture conditions vs Treg monocultures at day 14. The data were calculated from nine independent experiments with a two-sided Mann–Whitney U test with correction. Box plots indicate median (symbol within the box), 25th, 75th percentile (box), minimum and maximum values (whiskers). Source data are provided as a Source Data file.
culture conditions in terms of viability (≥90% for all cultures) and percentage of Helios (Fig. 1c), CTLA-4 (cytotoxic T cell antigen 4), CD127, heat shock protein 60 (HSP-60), HSP-70, HSP-90, CD31 and Nrp-1 (neuropilin 1) positive cells. No statistically significant differences were observed also in the expression intensity of these antigens (measured as MFI). The proportions of naive (Tn), central memory (Tcm) and effector memory (Tem) Tregs identified as CD4^+^FoxP3^+^CD45RA^+^CD62L^+, CD4^+^FoxP3^+^CD45RA^−^CD62L^+^ and CD4^+^FoxP3^+^CD45RA^−^CD62L^−^ cells, respectively were also evaluated (Fig. 1e). Direct and indirect contact with ASCs resulted in increased frequencies of Tem cells as compared with Tregs expanded simultaneously in monocultures (MW; \( p = 7 \times 10^{-3} \) for
**Fig. 2 Direct and indirect Treg contact with allogeneic ASCs increases their potential for suppression of autologous Tconv proliferation.**

**a** Histograms from 1 representative experiment depict dilution of violet proliferation dye 450 (VPD-450) by proliferating Tconvs in presence of Tregs from various culture conditions in Treg:Tconv ratios of 2:1 (left panel), 1:1 (middle panel) and 1:2 (right panel). Histograms for negative (unstimulated Tconvs without Tregs) and positive control (stimulated Tconvs without Tregs) are also presented. **b** Comparison of proliferation indexes (PI) of Tconvs in the presence of various proportions of autologous Tregs derived from indicated culture conditions is shown for 6 independent experiments. VPD-450 stained unstimulated and stimulated Tconvs served as negative and positive controls, respectively. Squares, circles, filled and empty triangles correspond with the proliferation suppression effect exerted by Tregs expanded in standard monocultures, monocultures supplemented with ASC-derived SNs, direct and indirect cocultures, respectively. The differences were calculated with a two-sided Mann-Whitney U test with correction. *p < 0.05 for direct cocultures vs monocultures, **p < 0.05 for comparisons between Tregs from monocultures supplemented with ASC-derived SNs vs Tregs from direct or indirect cocultures are marked with *** and ****, respectively. In the whisker plots the median is indicated by the symbol between the whiskers. The lower and upper whiskers indicate the minimum and maximum values, respectively. Source data are provided as a Source Data file.

**Tregs expanded in direct and indirect contact with ASCs suppress proliferation of conventional T cells (Tconvs) more efficiently than Tregs from monocultures.** Suppression of conventional T cell (Tconvs) proliferation is one of the crucial mechanisms used by Tregs to maintain immune homeostasis. Therefore, Tregs derived from monocultures and those conditioned by direct and indirect contact with ASCs were tested for their potential to suppress the proliferation of autologous Tconvs. The intensity of Tconv proliferation was measured as proliferation index (PI) which refers to an average number of divisions of responding cells. Thus, a stronger immunosuppressive potential of Tregs will result in a lower PI of Tconvs. The gating strategy for the analysis of Tconv proliferation and the calculation of PI is depicted in Supplementary Fig. 2. Tregs derived from direct and indirect cocultures with ASCs showed strikingly stronger suppression of proliferation of Tconvs (lower PI of Tconvs); as compared with Tregs not exposed to allogeneic ASCs. The differences between suppression of Tconv proliferation by Tregs preconditioned in the cocultures and Tregs from the standard monocultures were statistically significant for all studied Treg:Tconv ratios (MW; comparison between direct cocultures vs monocultures and indirect cocultures vs monocultures for 2:1, 1:1 and 1:2 Treg:Teff ratios were as follows: p = 2 × 10^-3 and p = 8 × 10^-3; p = 8 × 10^-3 and p = 0.02; p = 2 × 10^-3 and p = 0.02, respectively; Fig. 2). Interestingly, culture supplementation with ASC-derived SNs did not replicate the effects observed in indirect cocultures. Tregs treated with ASC-derived SNs did not promote a higher suppression of Tconv proliferation as compared with Tregs from standard monocultures and were less potent inhibitors than Tregs conditioned in direct cocultures with ASCs (MW; 2:1 p = 0.01, 1:1 p = 0.17, 1:2 p = 0.03; Fig. 2). The PI of Tconvs in the presence of the Tregs expanded in direct cocultures was 22.15, 19.63 and 18.3% (median) lower for 2:1, 1:1 and 1:2 Treg:Tconv ratios, respectively as compared with the values observed for Tregs expanded in standard monocultures. For Tregs conditioned in indirect cocultures vs those from monocultures this decrease was as follows: 15, 19.63 and 21.15%.

Direct and indirect contact with ASCs enhances Treg potential for generation of extracellular adenosine. Degradation of extracellular ATP and AMP (eATP and eAMP, respectively) leads to the accumulation of immunosuppressive ADO in the extracellular milieu (eADO). As in the canonical pathway this process takes place due to the activity of adenosinergic ectoenzymes CD39 and CD73. Therefore we compared the frequency of CD39^+ and CD73^+ cells within the Treg population in different culture conditions. However, the percentage of CD39^+ Tregs reached nearly 100% in all cultures and no statistically significant differences were found. The intensity of CD39 expression by CD39^+ Tregs was also comparable for cells derived from monocultures and cocultures (Fig. 3a). By contrast, the frequency of CD73^+ Tregs was higher in direct and indirect cocultures, than in monocultures (MW; p = 0.02 for both comparisons; Fig. 3b). In addition, an increase in the % of CD73^+ cells was observed as a function of time when Tregs from monocultures, direct and indirect cocultures at day 14 after isolation were compared with Tregs at day 7 that preceded coculture experiments (MW; p = 0.02, p = 3 × 10^-3, p = 1 × 10^-3, respectively; Fig. 3b). The highest intensity of CD73 expression was observed for Tregs derived from indirect cocultures and was significantly higher compared with Tregs obtained from standard monocultures (MW; p = 0.01; Fig. 3b).

The most significant differences between the culture conditions were identified in the activity of CD39 and CD73. Tregs conditioned with direct and indirect contact with ASCs as well as those from monocultures supplemented with ASC-derived SNs degraded significantly more eATP, than Tregs from standard monocultures (MW, p = 0.02 for all comparisons; Fig. 3c). Notably, however, Tregs from direct cocultures were characterized by a strikingly higher potential for eATP degradation as compared with cells from the other tested conditions (MW; p = 0.02 for all comparisons). Tregs expanded in direct and indirect contact with ASCs as well as those exposed to ASC-derived SNs degraded significantly more eAMP, than Tregs from standard monocultures (MW, p = 0.02 for all comparisons). In addition, direct and indirect communication with ASCs stimulated Treg-dependent eAMP degradation significantly more than the transfer of ASC-derived SNs into Treg cultures (MW; p = 0.02 for both comparisons; Fig. 3c). In general, 1 × 10^6 Tregs conditioned by direct contact with ASCs degraded 44% (median) more nmol of eATP and 402% (median) more nmol of eAMP after 1 min, relative to Tregs expanded in standard monocultures. These values were equal to 26% and 244.4%, respectively, when the potential for eATP and eAMP degradation by Tregs from indirect cocultures were compared with the results obtained for standard monocultures. At the same time, no differences were observed between the culture conditions in terms of eADO degradation (Fig. 3c). These data indicate that bidirectional Treg-ASC communication (notably resulting from direct contact) enhances the immunoregulatory potential of Tregs. Elimination of proinflammatory eATP and eAMP is known to prevent from
life-threatening multiple organ dysfunction in systemic inflammatory response syndromes (SIRS)\(^3\), while eADO, the product of eATP and eAMP degradation was shown to induce immune tolerance\(^3\)). In addition, inverse correlations were observed between proliferation of Tconv in the presence of Tregs and in Treg-mediated degradation of eATP and eAMP (Spearman’s rank correlation, SC; \(R = -0.43, p = 2 \times 10^{-3}\) and \(R = -0.6, p = 8 \times 10^{-6}\), respectively). No similar correlations were observed for eADO (Fig. 3d; \(R = -0.1, p = 0.46\)). These data suggest that, degradation of eATP and eAMP is an important mechanism of Treg-mediated suppression of conventional T cell proliferation.
Tregs uptake plasma membrane and mitochondria from allogeneic ASCs. Recent studies show that intercellular communication is a complex process that may involve uptake of plasmalemma fragments with surface antigens^{38,39}, as well as mitochondrial DNA transfer via exosomes^{40}. Thus, in the present study we performed a set of cocultures to determine whether Tregs can uptake fragments of plasma membrane, cytosol and whole mitochondria from allogeneic ASCs. The gating strategy for organelle transfer analysis is depicted on Supplementary Fig. 4. The membrane and mitochondria uptake was completely abolished when both cell types were separated from each other with culture inserts with a membrane pore size of 0.4 μm. Thus statistically significant differences between direct and indirect cocultures in terms of plasmalemma and mitochondria uptake were observed (MW; p = 0.02 for transport of both elements; Fig. 4). The presence of 18-β-glycyrrhetinic acid (18β) and latrunculin A (LA) a gap junction blocker^{41} and actin filament inhibitor that blocks tunnelling nanotube formation^{42}, respectively, did not diminish substantially the transfer in direct cocultures. Thus, no statistically significant differences in terms of plasmalemma and mitochondria uptake were found between Tregs cultured in direct contact with ASCs in the presence or absence of 18β or LA.

Majority of Tregs internalize active allogeneic mitochondria and the process correlates with HLA eplet mismatch load. As the uptake of ASC-derived mitochondria by Tregs was a common and extensive phenomenon we decided to define the functional status of these mitochondria. Thus, in further experiments ASCs were labelled simultaneously with MIG and CM-H2XRos dyes. CM-H2XRos is a nonfluorescent compound that becomes fluorescent upon oxidation into CM-Xros. Thus, MIG staining served for evaluation of the whole mass of internalized mitochondria, while CM-Xros enabled to distinguish mitochondria that were undergoing aerobic respiration (MIG+CM-Xros+) from non-functional ones (MIG+CM-Xros−). The gating strategy for these experiments is shown in Supplementary Fig. 3. In the current study, we found that >85% of mitochondria internalized by Tregs were functional. The median proportion of functional mitochondria internalized by Tregs was 96.3, 86.7 and 95.2 % for direct Treg-ASC cocultures without inhibitors and in the presence of 18β or LA, respectively (Fig. 5). Thus, statistically significant differences in the uptake of respirating mitochondria were observed for these three culture conditions as compared with both monocultures and indirect cocultures (MW; p = 5 × 10−4 for Treg-ASC direct cocultures without inhibitors; p = 2 × 10−3 for Treg-ASC direct cocultures with 18β; and p = 1 × 10−3 for Treg-ASC direct cocultures with LA). In addition, for all direct cocultures a statistically significant difference between uptake of functional and non-functional mitochondria was observed (MW; p = 5 × 10−4, p = 7 × 10−3 and p = 2 × 10−3 for the direct coculture without inhibitors and those with 18β and LA, respectively). The control experiments confirmed that the frequency of active mitochondria in ASCs was decreased after direct coculture with allogeneic Tregs (MW; p = 0.03 for % of active mitochondria in ASCs before vs after the direct coculture with Tregs; Supplementary Fig. 4).

To keep Treg proliferation and survival the culture medium was supplemented with IL-2. Tregs and ASCs used in the cocultures were obtained from unrelated donors and the medium in the cocultures was rich in proinflammatory cytokines secreted by Tregs and ASCs (see the analysis of culture SNs). This methodology resulted in the induction of HLA-DR expression on ASCs. Therefore, we decided to determine impact of HLA class I and class II incompatibility on mitochondria transfer. High-resolution typing of HLA-A, -B, -C, -DRB1 and -DQBI alleles for all Treg and ASC donors was performed (Supplementary Table 1 and Supplementary File 1). This analysis revealed that each Treg-ASC pair was mismatched in the HLA class I and class II antigens. In addition, using the HLAMatchmaker algorithm we determined the HLA eplet mismatch load for each pair of tested HLA alleles and found that it was higher for HLA class I, than for class II antigens for all studied Treg-ASC pairs (Table 1). Further analysis revealed that internalization of active mitochondria in the absence of transport inhibitors correlated positively with HLA-C and HLA-DRB1 eplet mismatch load (SC; R = 0.75, p = 0.08 and R = 0.89, p = 0.01, respectively; Fig. 5g).

Treg mitochondria uptake is HLA dependent. To determine whether Treg-ASC mitochondria transfer depends on HLA expression, we used the K562 cell line for the control experiments. K562 is a HLA-negative human erythromyeloblastoid leukemia cell line^{43}. Thus, it is a well-defined tool to study the impact of HLA in allogeneic models. To distinguish K562 cells from Tregs in the direct cocultures the cells were labelled with anti-HLA-ABC antibodies just before the analysis. In addition, like ASCs, K562 cells are significantly bigger (∅ of 17 μm) and more intracellularly complex cells than Tregs (∅ of 6–8 μm). As higher cell granularity results in higher side scatter (SSC) values in flow cytometry analysis, K562 were identified as HLA-ABC−SSC\text{High}, while Tregs were gated as HLA-ABC−SSC\text{Low} cells (Fig. 6a). Flow cytometry analysis (Fig. 6a, b) and confocal microscopy imaging (Fig. 6c) revealed no mitochondria transfer.
from K562 cells to Tregs. Subsequently, to determine whether mitochondria transfer from K562 cells to Tregs was abolished by lack of HLA expression or resulted from different features of K562 cells, we performed experiments with anti-HLA class II blocking antibody (clone IVA12). These experiments indicated that blocking HLA class II molecules on allogenic ASCs significantly suppresses mitochondria transfer from ASCs to Tregs. Analysis of two independent Treg-ASC pairs in duplicates revealed that lack of HLA class II antigen availability on ASCs limited the mitochondria uptake for ≥80% (Fig. 6d).
**Fig. 4 Tregs uptake plasmalemma and mitochondria, but not cytokos from allogeneic ASCs when present in direct cell-to-cell contact.** Plasmalemma, mitochondria and cytokos of allogeneic ASCs were stained with a Vybrant DJD (DID), b mitotracker green (MIG) and c calcein violet (CV), respectively. Unstrained Tregs cultured separately served as a negative control (Solo-MIX, n = 4) and were mixed with stained ASCs immediately before the analysis with flow cytometry to show Treg autofluorescence and fluorescence of labelled ASCs on the same graph. Simultaneously, stained ASCs were cocultured with unstrained Tregs for 72 h in direct (Direct, n = 4) and indirect (Indirect, n = 4) contact. In addition, the effect of 18β-glycyrrhetinic acid (18β GA, n = 4 and 18β 200 μM, n = 4) and latrunculin A (LA 2 μM, n = 4 and LA 10 μM, n = 4) inhibitors of gap junction and tunnelling nanotube formation, respectively was tested in direct cocultures. Graphs depict the percentage of Tregs that internalized ASC-derived cellular elements. Histograms from one representative experiment are shown. Red and blue histograms represent the fluorescence of unstrained Tregs and stained ASCs, respectively. Cellular element uptake is visible as an increase in the fluorescence of unstrained Tregs after coculture with labelled ASCs. The frequency of Tregs that internalized ASC-derived element is shown on each histogram. Data for four independent experiments were calculated using a two-sided Mann-Whitney U test with correction. *p < 0.05 for direct cocultures with and without inhibitors vs monocultures and indirect cocultures. In all boxplots the median is indicated by the symbol within the box, lower and upper bounds of the boxes correspond with the 25th and 75th percentiles. The lower and upper whiskers indicate the minimum and maximum values, respectively. Source data are provided as a Source Data file.

Direct and indirect contact of Tregs and ASCs is associated with significantly different cytokine profile and depends on HLA compatibility. To determine whether HLA incompatibility impacts on the cytokine milieu in the Treg-ASC cocultures we measured concentrations of 50 various cytokines and growth factors from the direct and indirect cocultures and correlated them with the number of HLA class I and class II eplet mismatch load. Analysis of SNs collected after 24 h and 48 h from Treg and ASC monocolonies revealed striking statistically significant differences in the concentration of all 50 analysed mediators as compared with the cocultures (Supplementary Table 2). For further analysis the factors were roughly grouped into pro- and anti-inflammatory cytokines, cytokines stimulating growth and survival of lymphoid and myeloid cells, chemokines and growth factors. Surprisingly, the concentration of all types of the mediators was significantly increased in direct Treg-ASC cocultures. SNs collected from Treg and ASC monocolonies were characterized by relatively low levels of the analysed factors, while the intermediate concentrations were characteristic for indirect Treg-ASC cocultures (Fig. 7a). The further analysis defined the impact of HLA-DR and HLA class I eplet mismatch load on cytokine secretion and Treg proliferation in the cocultures (Supplementary Table 2 and Fig. 7). HLA-B eplet mismatch load correlated positively with the concentration of the following cytokines in the cocultures: IL-3, IL-9, IL-10, IL-17A, IL-17E. IL-22 and M-CSF (macrophage colony-stimulating factor). HLA-C eplet mismatch load correlated positively only with IL-6 levels. The strongest impact on cytokine secretion resulted from eplet mismatches in HLA-DRB1. They correlated positively with the levels of the following mediators: SCAD01L, FGF-2 (fibroblast growth factor 2), G-CSF (granulocyte colony-stimulating factor), GM-CSF (granulocyte-macrophage colony-stimulating factor), IFN-α2 (interferon α2), IFN-γ, IL-1α, IL-5, IL-9, IL-17A, IL-18, IL-27, MIP-1α (macrophage inflammatory protein-1α), MIP-1β, PDGF-AA (platelet-derived growth factor AA), TGFα (transforming growth factor α) and TNF-α (tumour necrosis factor α; Supplementary Table 2).

In addition, the concentrations of IL-6, FGF-2, G-CSF, IFN-α2, IL-17E, IL-18, IL-22 and TGF-α were significantly higher in indirect cocultures, than in both Treg and ASC monocolonies and their levels correlated with HLA mismatch eplet load between ASC and Treg donors (Supplementary Table 2).

Treg proliferation in direct and indirect contact with allogeneic ASCs correlates negatively with the levels of proinflammatory cytokines and HLA eplet mismatch load. Previous studies demonstrated that the presence of proinflammatory mediators suppresses Treg proliferation and function44. Therefore, in the current study we also analysed Treg proliferation potential in the context of cytokine milieu and HLA eplet mismatch load in the given monocultures and cocultures.

Although Tregs in direct cocultures preferentially proliferated on ASC surface (Movie S1), we found that they exhibited reduced cell divisions compared to the cells from monocultures and Tregs from indirect cocultures (MW; p = 0.03 and p = 6 × 10−3, respectively Fig. 7b). The highest proliferative potential characterized the latest culture condition and was significantly higher as compared with the standard monoculture (MW; p = 3 × 10−3; Fig. 7b). For better visualization of the differences in Treg proliferation between cocultures and monocolonies, the absolute Treg numbers were converted into INDEX of Treg proliferation (Fig. 7). The INDEX of Treg proliferation was calculated with a different algorithm, than the proliferation INDEX of Tconv5 discussed in the proliferation suppression assay. The detailed descriptions are given in the “Methods” section.

In addition, after 48 h the direct cocultures exhibited significantly higher levels of the following 21 cytokines as compared with indirect cocultures (Supplementary Table 3): IL-22, MCP-3 (monocyte chemotactic protein-3), IL-12p70, EGF, eotaxin, IL-12p40, IL-6, MIG (monokine induced by interferon-γ), IL-1β, IL-15, IL-7, IP-10 (IFN-γ-induced protein 10), VEGF-A (vascular endothelial growth factor A), IL-8, IL-18, RANTES (regulated upon activation, normal T cell expressed and secreted), fractalkine, MCP-1, MDC/CCCL22 (macrophage-derived chemokine), FLT3L (FMS-like tyrosine kinase 3 ligand) and PDGF-AB/BB. All these mediators correlated negatively with the Treg proliferation potential and are listed in order of the strength of the correlation in Supplementary Table 3. The most significant correlations (R ≤ −8) were found for IL-22, MCP3, IL-12p70, EGF, eotaxin, IL-12p40 and IL-6 and are shown in Fig. 7b. In addition we observed negative correlation between Treg proliferation and eplet mismatch load for HLA-DRB1 (SC; R = −0.47, p = 0.04; Fig. 7c).

**Discussion** In this study, we demonstrate that both direct and indirect contact with allogeneic ASCs improves therapeutic potential of Tregs. In addition, we show that Tregs internalize active mitochondria and plasma membrane of allogeneic ASCs when present in direct cell-to-cell contact and thus improve their immunosuppressive activity. Functional mitochondria are the most extensively transferred cellular elements from ASCs to Tregs and their uptake correlates positively with HLA-C and HLA-DRB1 eplet mismatch load. Moreover, our experiments with HLA-null K562 cell line and blocking anti-HLA class II antibody revealed that Treg uptake of allogenic mitochondria depends on HLA expression on donor cells. With this study we also demonstrate the significance of Treg-ASC communication with soluble factors and...
Table 1 HLA class I and class II eplet mismatches.

| No. of Treg-ASC pair | HLA-A eplet mismatches (HLA-A eplet mismatch load) | HLA-B eplet mismatches (HLA-B eplet mismatch load) | HLA-C eplet mismatches (HLA-C eplet mismatch load) | HLA-DRB1 eplet mismatches (HLA-DRB1 eplet mismatch load) | HLA-DQB1 eplet mismatches (HLA-DQB1 eplet mismatch load) |
|----------------------|---------------------------------------------------|---------------------------------------------------|---------------------------------------------------|---------------------------------------------------|---------------------------------------------------|
| 1. 43Q, 62GER, 62GE, 62GK2, 107W, 127K, 144TKH, 145KHA | 41T, 80I, 80I + 69TNT, 80I + 90A, 82LR, 82LR + 90A, 82LR + 138T, 82LR + 144QR, 82LR + 145R, 82LR + 145RA, 131S + 163T, 156DA, 158T | 80K + 14R | 11TS, 74R, 77N | 45GE3, 77R |
| 2. 62QE + 56G, 76ESL, 82LR + 138M | 131S + 163T, 158T | 2H, 80K | 13FE, 47F, 56EDR11, 56EDR11, 57DE, 57DEDP, 96EV | 74R, 77N | 45GE3, 77R |
| 3. 66NV, 16ID | 41T, 80I, 80I + 69TNT, 80I + 90A, 82LR + 138T, 156DA, 163LS/G | 138K + 177KT, 193PL3, 267QE | 13FE, 47F, 56EDR11, 56EDR11, 57DE, 57DEDP | 74R, 77N | 45GE3, 77R |
| 4. 62EE, 65GK, 82LR + 138M, 144KR + 127K | 65QIA, 65QIA + 76ESN, 69AA + 65QIA + 76ESN, 131S + 163T, 158T, 163EW, 163EW + 66I, 163EW + 73TE, 180E | 193PL3, 267QE | 47F, 56EDR11, 56EDR11, 57DE, 57DEDP | 13FE, 47F, 56EDR11, 56EDR11, 57DE, 57DEDP | 74R, 77N |
| 5. 62EE, 65GK, 80I, 80I + 90A, 82LR + 138M, 144KR, 144KR + 127K, 144KR + 151H, 166DG | 41T | 2H, 65QIA + 76ESN, 80I, 80I + 69TNT, 80I + 90A, 82LR + 144QR, 82LR + 145R, 82LR + 145RA, 158T, 163EW, 163EW + 66I, 163EW + 73TE, 180E | 13FE, 47F, 56EDR11, 56EDR11, 57DE, 57DEDP | 74R, 77N | 45GE3, 77R |
| 6. 43Q + 62GER, 62GE, 62GK2, 79GT + 90D, 107W, 144TKH, 145KHA, 151AHA, 163R, 163RW | 44RT, 44RT + 69TNT, 65QIA, 69AA, 69AA + 65QIA + 76ESN, 69AA + 65QIA + 76ESN, 80I, 80I + 69TNT, 82LR + 138T, 82LR + 144QR, 82LR + 145R, 82LR + 145RA, 131S + 163T, 156DA, 158T, 163EW, 163EW + 66I, 163EW + 73TE, 180E | 41T | 2H, 65QIA + 76ESN, 80I, 80I + 69TNT, 80I + 90A, 82LR + 144QR, 82LR + 145R, 82LR + 145RA, 158T, 163EW, 163EW + 66I, 163EW + 73TE, 180E | 13FE, 47F, 56EDR11, 56EDR11, 57DE, 57DEDP | 74R, 77N |
| 7. 66NV, 151AHA, 163RW | 44RT, 44RT + 69TNT, 65QIA, 69AA, 69AA + 65QIA + 76ESN, 69AA + 65QIA + 76ESN, 80I, 80I + 69TNT, 80I + 90A, 82LR + 144QR, 82LR + 145R, 82LR + 145RA, 158T, 163EW, 163EW + 66I, 163EW + 73TE, 180E | 193PL3, 267QE | 47F, 56EDR11, 56EDR11, 57DE, 57DEDP | 13FE, 47F, 56EDR11, 56EDR11, 57DE, 57DEDP | 74R, 77N |
| 8. 62QE + 56G, 66NV, 79GT + 90D, 90D, 138MI, 138MI + 79GT, 144KR, 144KR + 151H, 151AHA, 163R, 163RW | 44RT, 44RT + 69TNT, 65QIA, 69AA, 69AA + 65QIA + 76ESN, 80I, 80I + 69TNT, 80I + 90A, 82LR + 144QR, 82LR + 145R, 82LR + 145RA, 158T, 163EW, 163EW + 66I, 163EW + 73TE, 180E | 44RT, 44RT + 69TNT, 65QIA, 69AA, 69AA + 65QIA + 76ESN, 80I, 80I + 69TNT, 80I + 90A, 82LR + 144QR, 82LR + 145R, 82LR + 145RA, 158T, 163EW, 163EW + 66I, 163EW + 73TE, 180E | 193PL3, 267QE | 47F, 56EDR11, 56EDR11, 57DE, 57DEDP | 74R, 77N |
| 9. 62Q + 62GER, 62GE, 62GK2, 62Q, 62Q, 62Q + 144QR, 80TLR, 82LR, 82LR + 90A, 82LR + 138T, 82LR + 144QR, 82LR + 145R, 82LR + 145RA, 131S + 163T, 156DA, 158T | 41T | 73AN, 90D | 25Q3, 7V, 70D, 78V2, 98E | 104A, 181M | 74R, 77N |

Antibody verified eplet mismatches in HLA-A, -B, -C, -DRB1 and -DQB1 antigens between Treg and ASC donors are listed and HLA eplet mismatch load for each studied allele pair is given in brackets.
its dependency on HLA mismatches. These data all together shed light on our understanding of intercellular communication and mechanisms that might be involved in allograft tolerance development.

Despite the fact that Tregs and mesenchymal stem cells (MSCs) are cells of distinct origin, they both demonstrate strong immunosuppressive potential and have been used in clinical trials for the induction of tolerance towards self-tissues and non-self-allografts. Therefore, in the current study we aimed to exploit the potential of adipose tissue-derived MSCs (ASCs) to elaborate improved system of Treg expansion. Our experiments confirmed initial hypothesis that direct contact with allogeneic ASCs enhances the immunosuppressive activity of Tregs. However, we observed that also Tregs from indirect cocultures exhibited a significantly stronger capacity for suppression of conventional T cell (Tconvs) proliferation, than Tregs from standard monocultures and monocultures supplemented with ASC-derived SNs. Moreover, Tregs conditioned in both cocultures exhibited a significantly higher potential for eATP and eAMP degradation that leads to the accumulation of tolerance-inducing eADO as compared with Tregs from other tested culture models. Interestingly, the transfer of SNs derived from ASC monocultures could not replicate the biological effects observed in indirect cocultures where ASCs and Tregs were separated from...
each other with a cell impermeant membrane. In these two culture conditions, Tregs were exposed to an ASC-derived cytokine cocktail and in both cultures Tregs could not communicate with ASCs via surface receptors, but the proportions of the mediators present in these two cultures differed significantly. Our data suggest that the cells communicated bidirectionally via secreted cytokines and could therefore sense the presence of the other cell type via a pattern of soluble mediators present in their milieu. The concentration of IL-6, FGF-2, G-CSF, IFN-α2, IL-17E, IL-18, IL-22, IL-27 and TGF-α was significantly higher in indirect cocultures, than in both Treg and ASC monocultures. Moreover, the levels of these factors correlated with HLA eplet mismatch load between ASC and Treg donors. This implies that even devoid of direct contact Tregs and ASCs were able to distinguish cells of non-self-origin and responded with the secretion of a specific cytokine signature. Nevertheless, direct cell-to-cell interaction had the strongest effect on Treg function which is in accordance with previous studies on cell communication34,46,47. Mechanisms of direct cell interactions are poorly understood and most of the previous studies investigated just surface receptor induced signalling48,49. Just recently, it was reported that cell-to-cell contact may also result in trogocytosis, the process of antigen removal from the surface of the target cell and its internalization. Such a phenomenon was reported for Treg interactions with dendritic cells (DCs) and was associated with the uptake of surface molecules involved in the formation of the immune synapse such as the cognate peptide-MHC class II complexes (pMHCIIs), CD86, ICOS-L (inducible co-stimulator molecule ligand) and PD-L2 (programmed cell death-ligand 2). By this mechanism, Tregs were able to prevent antigen presentation and activation of conventional T cells (Tconvs)38,39. However, in our study, the internalization of fragments of plasma membrane derived from ASCs was detected in a minority of Tregs. Surprisingly, mitochondria were the most extensively internalized cellular element by Tregs. In addition, the majority of these mitochondria were undergoing oxidative respiration, suggesting that the phenomenon may prevent apoptosis and/or degeneration of Tregs. Nevertheless, in our study the transfer of active mitochondria did not improve Treg proliferation and viability, but enhanced their immunoregulatory potential. Tregs that internalized ASC-derived mitochondria (direct cocultures) showed significantly higher stability of FoxP3 expression. These data are in accordance with the previous study of Khosravi et al. who demonstrated in a murine model that MSCs enhance Treg TSDR (Treg-specific demethylated region) demethylation in direct cocultures30. However, the most striking difference was found when the activity of CD39 - a triphosphohydrolase 1 was analysed. Tregs that internalized allogenic mitochondria exhibited a significantly higher potential for eATP degradation, than Tregs from other tested culture conditions, including those from indirect cocultures. High amounts of eATP are released into the extracellular space following tissue injury. When eATP binds to P2 receptors (virtually expressed on all immune cells) it induces cell activation and inflammatory responses37. Therefore, inhibition of the eATP mediated pathway halts lymphocyte activation and suppresses both Th1 and Th17 cells31. It has been also reported recently that a high potential for eATP degradation is crucial for the development of Treg-mediated operational tolerance after solid organ transplantation52. Therefore, these data suggest that uptake of allogenic mitochondria by Tregs might be one of the mechanisms crucial for the induction of graft tolerance.

Despite the fact that all of our cultures had relatively low numbers of CD73+ Tregs, as defined by flow cytometry, the cells from direct and indirect cocultures showed increased potential for eAMP degradation to eADO as compared with the monocultures. Interestingly, unlike for eATP and eAMP, Tregs from all conditions did not differ in terms of capacity for eADO degradation. Thus, direct and indirect contact with allogenic ASCs lead to the accumulation of eADO in the cultures. eADO limits inflammatory responses in order to avoid tissue damage and promote the healing process. It is also an immunosuppressive molecule capable of inhibiting the function of various immune cells including dendritic cells, monocytes/macrophages, T, B and NK cells. Thus, its accumulation in the extracellular space promotes graft survival and ameliorates autoimmune reactions35.

To better understand the mechanisms of interactions between Tregs and allogenic ASCs we performed a high-resolution typing of HLA-A, -B, -C, -DRB1, and -DQB1 loci for Treg and ASC donors. This method of HLA typing is a standard procedure performed before solid organ and bone marrow/hematopoietic stem cell transplantation53,54, as donor-recipient matching is crucial for tolerance and long-term survival of the transplanted solid organs53 and minimizes the risk for acute GVHD in terms of bone marrow transplantation54. Subsequently, for defined HLA mismatches, we searched for eplet mismatch load (number of mismatches in functional epitopes) that determines immunogenicity of the given HLA incompatibility. Due to various eplet mismatch loads, certain HLA mismatches might be more immunogenic than the others resulting in an excessive response against foreign tissue55. In our model, all Treg-ASC pairs derived from HLA-mismatched donors. Therefore, we searched for a correlation between HLA incompatibility and mitochondria
Interestingly, we observed that the rate of allogeneic mitochondria incorporation by Tregs that resulted in their higher immunosuppressive potential correlated positively with HLA-C and HLA-DRB1 eplet mismatch load. Connection between these two HLA loci and Treg responses is in accordance with previous studies on maternal-foetal tolerance and with the dogma of HLA class II recognition by CD4⁺ T cell, including Tregs. Interplay between Tregs and HLA-C of allogeneic origin plays a significant role in maternal-foetal immune tolerance induction. Foetal HLA-C was shown to be a key molecule that can elicit immune-mediated foetal loss as it is specifically recognized by maternal T cells. However, HLA-C mismatched uncomplicated pregnancies are characterized by increased counts of functional decidual Tregs, compared to HLA-C matched pregnancies that prevent anti-foetal immune responses. Thus, Treg uptake of mitochondria from HLA-C mismatched cells can be one of the mechanisms triggering Treg-mediated foetal and allogenic graft tolerance. A similar scenario may be involved in Treg-induced tolerance of MHC class II mismatched allograft.

Taking into account all these observations, we decided to determine whether the process of mitochondria transfer can be affected by the absence of HLA antigens on donor cells. Thus, we used HLA-null K562 human erythromyeloblastoid leukemia cell line in the control experiments. Surprisingly, Tregs did not

---

**Diagram A:**
- Top: Scatter plot showing the correlation between HLA-ABC and Tregs.
- Middle: Flow cytometry histograms for Solo, Direct, and Indirect counts.
- Bottom: Bar graph showing mitochondria uptake.

**Diagram B:**
- Flow cytometry histograms for Solo, Direct, and Indirect counts.

**Diagram C:**
- Images showing fluorescence microscopy of Tregs.

**Diagram D:**
- Flow cytometry histograms for Solo, Direct, and Indirect counts.
internalize mitochondria from K562 cells. Both K562 and ASCs were allogenic cells, but unlike ASCs, the K562 cell line is negative for both HLA class I and class II antigens. To determine whether the lack of mitochondria transfer from K562 cells to Tregs resulted from features of K562 cells, other than the absence of HLA antigens, we performed experiments with blocking antibodies. Thus, we preincubated ASCs with an anti-HLA class II blocking antibody to mask HLA class II molecules on ASCs before the coculture. We decided to target HLA class II molecules, because they are crucial for Treg antigen-specific recognition. In addition, as was mentioned in the Results section, the presence of IL-2 and proinflammatory cytokines in the direct cocultures induced the expression of HLA class II molecules on ASCs. Thus, our in vitro culture environment mimicked to some extent the inflammatory response present in the body after graft transplantation. This experiment demonstrates that the masking of HLA class II molecules limits ASC-Treg mitochondria transfer by >80% and simultaneously suggests that HLA class I recognition is also involved in Treg-ASC interaction and mitochondria uptake by Tregs. These data are in accordance with the previous studies on HLA-C recognition by Tregs in the development of maternal-fetal tolerance. Thus, our study not only reveals a previously unknown mechanism of cell interaction based on mitochondria transfer, but also defines the role of HLA in this kind of communication. In addition, our experiments excluded the role of gap junctions and nanotubes in this transfer. At the same time, HLA-dependent mitochondria uptake and full inhibition of this process in indirect cocultures suggest that at least some mitochondria were transported in membranous carriers expressing HLA molecules.

Interestingly, the significance of mitochondrial energy metabolism for immunoregulatory activity of Tregs was reported previously by Beier and colleagues. The group observed that Treg activation led to the expression of genes important for oxidative phosphorylation (OXPHOS). Deletion of key regulators of OXPHOS such as Pgc1a or Sirt3 was found to diminish Treg suppressive function in vitro and in vivo. Thus, we can presume that the uptake of active allogenic mitochondria by Tregs can improve their energy production leading to enhanced immunosuppressive potential. Previous reports also suggested that high CD39 activity stimulates mitochondrial function and biogenesis. However, in the light of our data-enhanced CD39 activity seems to result from elevated mitochondrial activity, rather than its consequence.

Noteworthy, despite the fact that Tregs from direct cocultures were characterized by the highest immunosuppressive potential, they exhibited a lower proliferation rate, than Tregs from indirect cocultures. Our results suggest that these differences resulted from a significantly higher concentration of proinflammatory cytokines in direct cocultures. The strongest negative correlations between cytokine levels and Treg proliferation were found for MCP-3, IL-22, IL-12p70, EGF, eotaxin, IL-12p40 and IL-6. These data partially correspond with previous studies where IL-12, IL-6, TNF-α or MCP-3/CCL7 were reported to suppress Treg proliferation. However, it is interesting that there is a different cytokine communication between Tregs and ASCs when present in direct and indirect contact. The levels of nearly all measured proinflammatory cytokines, chemokines and growth factors were significantly higher in direct cocultures, than in the indirect communication model and higher in indirect cocultures, than in monocultures. These data indicate that Tregs recognize allogenic cells via both surface receptors and pattern of secreted soluble mediators. However, direct cell-to-cell contact had the strongest impact on the cytokine milieu and Treg function. These observations are in accordance with previous studies showing that Tregs can distinguish between self and non-self HLA and need activation via TCR-MHC class II signalling to exert immune suppression. In addition, it was reported that despite the ability of proinflammatory cytokines to suppress Treg proliferation, they can simultaneously enhance Treg immunosuppressive functions. The observations at least partially explain why Tregs derived from direct cocultures show the strongest immunosuppressive potential. In our study, direct cocultures were characterized by the highest concentrations of proinflammatory mediators and the highest frequencies of CD69 Tregs. Indirect cocultures also resulted in higher levels of proinflammatory cytokines and higher proportions of CD69 cells, as compared with monocultures, but these values were lower than observed for direct cocultures. Thus, the expression of CD69 reflected the levels of proinflammatory mediators in the cultures. These data are consistent with the study of Bremser and colleagues who demonstrated that induction of CD69 on Tregs is dependent on both- signalling via soluble factors (direct contact independent) and on TCR activation (direct contact dependent).

High Treg expression of CD69 seems to be of great importance for their immunoregulatory function. Compared to CD69+ cells, CD69+ Tregs were found to be more effective in maintenance of immune tolerance and only CD69+ Tregs were able to prevent the onset of inflammatory bowel disease (IBD) in mice. These data all together indicate that Treg-mediated suppression is a complex process where both HLA recognition and indirect communication via soluble factors play a role.
In summary, in the current study we demonstrate that direct or indirect coculture with allogenic ASCs is a promising approach for potentiation of the immunoregulatory properties of Tregs. Tregs are able to recognize HLA-mismatched cells and internalize their active mitochondria in HLA eplet mismatch load-dependent manner. Our observations will initiate further studies on these extremely interesting and complex mechanisms of cell communication.

**Methods**

**Ethical compliance.** All blood and adipose tissue donors gave written informed consent. Participation in the study was voluntary and no compensation was
provided to the donors. The study was conducted in accordance with the guidelines of the Declaration of Helsinki and under the protocol approved by the Independent Ethics Commission for Research of the Medical University of Gdańsk (agreement no. NKBiR/353/2011).

**Study design.** The aim of the study was to determine how direct and indirect contact with allogeneic ASCs affects the function of human Tregs and to analyse the mechanisms of such communications. Thus, we designed a set of culture conditions where Tregs were expanded in direct or indirect contact with ASCs. In addition, standard Treg monocultures and Treg monocultures supplemented with ASC-derived SNs were used as controls. To fully understand the nature of intercellular communications, we analysed the transfer of cellular elements from ASCs to Tregs and analysed Treg phenotype, proliferation and immunosuppressive activity after culture in the presence or absence of ASCs. We also analysed the impact of HLA eplet mismatch load on mitochondria transfer. Treg proliferation, function and cytokine milieu in the cocultures. Finally, we determined whether the presence or absence of certain molecules on donor cells may affect mitochondria uptake by human Tregs. For these experiments we performed a set of direct and indirect cocultures of Tregs with the HLA-null K562 human cell line. Before the initiation of the study we hypothesised that Tregs internalize cellular elements derived from allogeneic ASCs when cultured in direct cell-to-cell contact and that this process increases the immunosuppressive potential of Tregs. Nevertheless, after data analysis we observed that indirect Treg-ASC communication potentiates Treg function. Interestingly, we found that cellular elements that were the most extensively taken up by Tregs from allogeneic ASCs were mitochondria and that this phenomenon was HLA dependent. The minimal sample size necessary to achieve reliable measurements was adjusted individually for each type of analysis thus sample sizes differ between the experiments. The time points for data collection were specified in advance and adjusted for the particular type of analysis. No outliers were excluded. All cells used in the experiments were isolated from healthy human donors, while K562 cells are a commercially available human erythroleukemia cell line that was purchased from Merck (cat. 98121407-1V1).

**Cell culture.** Tregs and Tconvs were expanded in X-vivo20 culture medium (Lonza, cat. no. BE94-448Q) supplemented with 10% FBS and IL-2 (2 × 10^5 U/ml; Proleukin; Chiron). On day 0 of culture, magnetic beads coated with anti-CD3 and anti-CD28 antibodies (CTIS Dynabeads CD3/CD28; Invitrogen, cat. 40203D) were added to the cultures at a 1:0.6 cell:bead ratio. For the first 7 days Tregs were expanded in monocultures to obtain sufficient numbers for the coculture experiments and then distributed into various culture conditions. Tregs were expanded alone during the whole experiment (14 days) and were used as target cells in proliferation tests.

ASCs were cultured in 25 and 75 cm^2 flasks with vented caps (Corning Primaria) in 10% FBS LG-DMEM medium (PAA). When 80% confluence was reached the cells were harvested by trypsinization (0.025% trypsin/EDTA, Lonza, cat. CC-5012). Then, their potential for differentiation into osteoblasts, adipocytes, and chondroblasts under standard in vitro differentiation conditions was tested to confirm stem cell properties. Simultaneously, the remaining ASCs were seeded at density of ~7 × 10^5 cells/cm^2 into 75 cm^2 flasks (Corning Primaria) and cultured for further experiments. Both Treg and ASC culture media contained penicillin (10 000 U/ml)—streptomycin (10 mg/ml) solution (Sigma, cat. P0781) and the cells were expanded at 37 °C, 5% CO₂ and 90% humidity.

Seven days after Treg isolation Tregs and ASCs were harvested, suspended in X-vivo20 (10% FBS, 2000 U/ml IL-2) and seeded into 24-well plates (Corning Costar) in the following conditions: (1) Tregs (2 × 10^5 cells/well) control monoculture of Tregs, (2) ASCs Solo (3 × 10^5 cells/well)-control monoculture of ASCs, (3) ASCs SNs (3 × 10^5 cells/well)-monoculture of ASCs from which SN was collected daily and transferred into Treg culture (Tregs SNs), (4) Tregs SNs (2 × 10^5 cells/well)- Treg monoculture supplemented with SNs derived from ASCs (ASCs SNs), (5) Direct-coculture where Tregs (2 × 10^5 cells/well) and ASCs (3 × 10^5cells/well) were expanded together in the same culture well, (6) Indirect-coculture where Tregs (2 × 10^5 cells/well) and ASCs (3 × 10^5cells/well) were separated from each other with transwell inserts containing a membrane pore size of 0.4 μm. Thus, the direct cell-to-cell communication between ASCs and Tregs was prevented. However, the passage of soluble mediators and receptors secreted by the two cell types was not affected. As IL-2 is crucial for Treg survival, but is not produced by Tregs and ASCs [33, 71], it was added to the Treg monocultures and cocultures and consequently was also added to ASC monocultures to prevent discrepancies resulting from different initial IL-2 levels. After 7 days Tregs were harvested. The isolation procedure was placed in the Treg collection buffer from the direct cocultures to avoid contamination with ASCs. Each time Tregs from direct cocultures were tested for eventual ASC contamination before they were used for functional tests. For the cases where Tregs collected from the direct cocultures would contain >5% of ASCs a negative immunomagnetic selection of CD4 + T-cells was performed in the protocol. However, each time gentle collection Treg purity was ~99%.

K562 cells were cultured in X-Vivo20 culture medium (Lonza) supplemented with 10% FBS and antibiotics. The cells were used in the control experiments to study mechanisms of Treg mitochondria uptake.

**Direct ASC-Treg cocultures were recorded with a JuLi microscope (NanoEntek) for 33 h with 2-min intervals with ×40 objective lenses.**

**Phenotype analysis.** After reaching ~80% confluency ASCs in second or third passage were harvested and stained with monoclonal antibodies (mAb) directed against the following antigens: CD105 (eBioscience, cat. 47-1057-42), CD90 (eBioscience, cat. 12-0909-42), CD73 (eBiosciences, cat. 17-0739-42), CD44 (BD Biosciences, cat. 550144), CD34 (BD Biosciences, cat. 550131) and CD127 (BD Biosciences, cat. 558117). The post-sort purity of Tregs and Tconvs was approaching 100% [median-min (max): 98% (97%–99%)]. ASCs were isolated from fresh samples of adipose tissue (50–200 ml) obtained during liposuction procedures from nine healthy individuals (female 4, male 5) admitted to the Clinic of Plastic Surgery of University Clinical Centre of Gdańsk. As described before [33], after blood donation peripheral blood mononuclear cells (PBMC) were obtained by Ficoll/Uroplone gradient centrifugation (1260 g). Then, CD4 + T cells were isolated with negative immunomagnetic selection method with EasySep Human CD4 + T Cell Enrichment Kit (Stemcell Technologies, cat. 19052; selection purity 90–99%). Subsequently, CD4 + T cells were labelled with monoclonal antibodies (BD Biosciences, USA) specific for CD3 (cat. 558117), CD4 (cat. 561841), CD25 (cat. 555432), CD127 (cat. 560549), CD8 (cat. 347514), CD19 (cat. 340865), CD16 (cat. 338440) and CD14 (cat. 340660) antigens. The last 4 mAbs were conjugated with the same fluorochrome with the aim to cut-off in one step cytotoxic T cells (Tc), B cells, NK cells and monocytes, respectively. These cells were defined altogether in the sorting algorithm as lineage . Then, the cells were sorted with florescence activated cell sorter (FACS, Aria II, BD Biosciences) into the following phenotype of Tregs: CD3 + CD4 + CD25High CD127 − (Tregs) and Tconvs. As described before [33], it was added to the Treg monocultures and cocultures and consequently was also added to ASC monocultures to prevent discrepancies resulting from different initial IL-2 levels. After 7 days Tregs were harvested. The isolation procedure was placed in the Treg collection buffer from the direct cocultures to avoid contamination with ASCs. Each time Tregs from direct cocultures were tested for eventual ASC contamination before they were used for functional tests. For the cases where Tregs collected from the direct cocultures would contain >5% of ASCs a negative immunomagnetic selection of CD4 + T-cells was performed in the protocol. However, each time gentle collection Treg purity was ~99%.

K562 cells were cultured in X-Vivo20 culture medium (Lonza) supplemented with 10% FBS and antibiotics. The cells were used in the control experiments to study mechanisms of Treg mitochondria uptake.

The heat map depicts the secretion of various soluble mediators after 24 h and 48 h by Treg and ASC monocultures (Tregs Solo, n = 6 and ASCs Solo, n = 6, respectively) and direct and indirect cocultures (Direct, n = 6 and Indirect, n = 6, respectively). The listed factors were grouped into proinflammatory (PIC) and anti-inflammatory cytokines (A-IC), mediators stimulating growth and survival of lymphoid and myeloid cells (G&SLM), chemokines (CK) and growth factors (GF).

B Treg proliferation rate in relation to standard Treg monoculture (INDEX of Treg proliferation) in the presence or absence of ASCs is shown. The differences using data from nine independent experiments were calculated with two-sided Mann-Whitney U tests with correction. *p < 0.05 for direct cocultures vs mononcultures. **p < 0.05 for direct vs indirect cocultures. ***p < 0.05 for indirect cocultures vs monocultures. The most significant correlations between the proliferation of Tregs from direct and indirect cocultures together (n = 12) and cytokine levels in these cocultures are shown. The impact of ASC-Treg HLA-DRBI eplet mismatch load on the proliferation of Tregs from direct and indirect cocultures (n = 18). All correlations were calculated with Spearman’s rank correlation, R and p values are given. In all boxplots the median is indicated by the symbol within the box, lower and upper bounds of the boxes correspond with the 25th and 75th percentiles. The lower and upper whiskers indicate the minimum and maximum values, respectively. Source data are provided as a Source Data file.
Biosciences, cat. 555478), CD45 (BD Biosciences, cat.560777), and HLA-DR (BD Biosciences, cat.347402, 10 μl/test in 1:10 dilution). Then the cells were analysed by flow cytometry. BD Biosciences. Biocare (IVA12 clone; BIOZOL Diagnostica Vertrieb GmbH, cat. CBT-104701) that was reported to block HLA class II molecules75. The antibody was added at a final concentration of 10 μg/ml. 7 days after the coculture, the cells were washed twice, counted and used for the analysis of mitochondria transfer from K562 to Tregs in indirect and direct cocultures of unstained Tregs and unstained K562 cells. In addition, images were taken using a FLUOVIEW FV3000 objective lens. For each experiment stained and unstained Tregs and K562 cells were used as controls and analysed immediately after the staining, as well as after 72 h.

To evaluate the difference in % of active mitochondria within the whole mitochondria mass in ASCs before and after the direct cocultures ASCs were stained and analysed immediately before the culture. Simultaneously, control direct cocultures of unstained Tregs and K562 cells were performed and stained with MIG and CM-H2XRos after 72 h to evaluate ASC mitochondria activation status after the coculture. Then, the difference in the frequency of active mitochondria mass in ASCs before and after the culture was calculated was as number of divisions per number of cells that went into proliferation75. Results for the negative control (lack of stimulation) corresponded to the complete inhibition of Tconv proliferation, while results for the positive control to evaluate how contact with ASCs prevents or accelerates alterations in Treg phenotype.

Test of inhibition of Tconv proliferation by Tregs. At day 7 of the coculture Tconvs were stained with 2 μM of violet proliferation dye 450 (VPD-450; BD Horizon, cat. 562158) for 15 min. at 37 °C with agitation. Then, the cells were mixed with mouse anti-mouse Treg antibody. The next day the conditions in the following Treg:Tconv ratios: 1:1, 1:2, and 1:4 were used for the next 4 days at 37 °C in X-vivo20 medium supplemented with 10% FBS. The cell proliferation was measured as the proliferation index (PI) that is an average number of divisions that all responding cells have undergone since the initiation of the culture and was calculated as total number of divisions per number of cells that went into proliferation75. Results for the negative control (lack of stimulation) corresponded to the complete inhibition of Tconv proliferation, while results for the positive control (lack of inhibition) corresponded to proliferation of stimulated Tconvs in absence of Tregs.

Measurement of eATP, eAMP and eADO degradation. At day 7 after initiation of the cocultures Treg potential for eATP, eAMP, and eADO degradation was evaluated according to the previously described method74. In detail, the cells were distributed in 5 ml polystyrene tubes (1 × 106 Tregs/tube), washed twice with Hank’s balanced salt solution (HBSS) and preincubated in 1 ml of HBSS (pH 7.4) with HEPS (1.25 ml of HEPS/50 ml of HBSS) and glucose (0.05 g of glucose/50 ml of HBSS). At this step ENA (1 μM/100 μl HBSS) was also added into the tubes where degradation of eATP and eAMP was measured to block adenine deaminase activity and cells were incubated for 15 min. at 37 °C with gentle agitation. Subsequently, eATP, eAMP, or eADO were added at a final concentration of 30 μM and 70 μM of each SN was collected at 0, 5, 15 and 30 min time points. During the 7 days of Treg cultivation, the cells were kept at 37 °C and gently agitated. Each time the collection of SNs was preceded by a centrifugation step (2 min, 450 g) to eliminate the risk of cell collection that could affect the final results. The SNs were frozen immediately and stored at −80 °C until the analysis of the concentration of nucleotides and their catabolites was analysed with reverse-phase HPLC. The chromatographic system consisted of a Thermo Finnigan Surveyor autosampler and MS pump with UV6000RP Detector (Thermo Finnigan). An analytical column (150 × 2.1 mm) Kinetex C18 with a 2.6-μm particle size (Phenomenex) was used. The following chromatographic conditions were applied: buffer A was 122 mM sodium phosphate, 26.4 mM sodium hydrogen phosphate, and 150 mM potassium chloride; buffer B was a 15% (v/v) acetonitrile in buffer A. The amount of buffer B changed from 0% to 1% in 0.1 min, 3% in 3.3 min, 35% in 7.3 min, 100% in 9 min to 11.5 min, and 0% B in 11.6 min. The reequilibration time was 3.4 min, resulting in a cycle time of 15 min between injections. The detection limit was 200 μM, and the injection volume of injection was 20 μl. The analytical column was maintained at 23 °C. Peaks were monitored by absorption at 254 nm. Data were collected and processed by Thermo Scientific Xcalibur Software (v.2.0, ThermoScientific). The activity of eATP, eAMP, and eADO degrading enzymes was measured in nmol/min/× 106 cells and presented as INDEXes. The values shown are means ± SEM and thus set to 100% for each experiment. Consequently, the rates of eATP, eAMP, and eADO hydrolysis by Tregs cultured in all other tested conditions were converted accurately and are shown as % in relation to the standard.

Transfer of cellular elements. To analyse plasmamela, cytosol and mitochondria transfer ASCs were stained in warm (37 °C) PBS (1 × 106 cells/ml) with Vybrant® DIO Cell-Labeling Solution (Invitrogen, cat. V-22887; 1/50 dilution and 1 μg/ml), Calcein Violet AM (CV; Invitrogen, cat. C34858; 2.5 μM) and MitoTracker Green FM (MIG; Invitrogen; cat. M7514; 200 nM), respectively as recommended by the manufacturer. After a 30 min. incubation at 37 °C with agitation the cells were washed twice, counted and used for the analysis of cellular element transfer from ASCs to Tregs in indirect and direct cocultures in the presence or absence of various concentrations of 18β (Sigma, cat. G10105-10G; 10 and 200 μM) and LA (Sigma, cat. L1561-100UG; 2 and 10 μM). The time-course experiments demonstrated that 6 h is the minimal time required for ASC plasmamela and mitochondria uptake by Tregs. However, the 72 h time point was defined as the optimal time to perform the experiments. Longer coculture did not increase the rate of cellular element transfer. After 72 h, the cells were collected and labelled with anti-CI015 antibodies (eBioscience, cat. 47-1057-02) to distinguish CI015ASCs from ASCs. For each experiment stained and unstained Tregs and ASCs were used as controls and analysed immediately after staining, as well as after 72 h.

To evaluate the transfer of active and inactive mitochondria ASCs were stained in warm PBS (1 × 106 cells/ml) with MIG (200 nM) and MitoTracker Red CM-H2XRos (Invitrogen, cat. M7513; 500 nM). CM-H2XRos is a nonfluorescent compound that becomes fluorescent upon oxidation into CM-XR0s. Unlike MIG, CM-XR0s is accumulated in mitochondria in membrane potential dependent manner. Thus, double MIG and CM-H2XRos labelling serves for evaluation of the whole mitochondria mass (M) and discrimination of inactive (M′) and active (M″) mitochondria that undergo aerobic respiration. After a 72 h incubation on the cell culture plate the cells were washed twice and used for the analysis of active and inactive mitochondria transfer from ASCs to Tregs in indirect and direct cocultures in the presence or absence of 18β (200 μM) and LA (10 μM). After 72 h the cells were collected, labelled with anti-CI015 antibodies and analysed by flow cytometry (LSRFortessa, BD Biosciences). In addition images were taken using a confocal microscope (Zeiss, Germany) mounted on a microscope AxioslumerZZ (Zeiss, Germany) with transmitted-light illumination (DIC as a contrast) in the third channel with ≤20 objective lenses. For each experiment stained and unstained Tregs and ASCs were used as controls and analysed immediately after the staining, as well as after 72 h.

To evaluate the difference in % of active mitochondria within the whole mitochondria mass in ASCs before and after the direct cocultures ASCs were stained and analysed immediately before the culture. Simultaneously, control direct cocultures of unstained Tregs and K562 cells were performed and stained with MIG and CM-H2XRos after 72 h to evaluate ASC mitochondria activation status after the coculture. Then, the difference in the frequency of active mitochondria mass in ASCs before and after the culture was calculated.

In addition, control cocultures of Treg cells with HLA-negative human erythromyeloblastoid K562 cell line (Merck, cat. 89121407-1VL) were performed to elucidate the role of HLA expression in allogenic mitochondria uptake. As described earlier, for the 7-day Treg-ASC culture, the cells were harvested and counted. Simultaneously, K562 cells were stained in warm (37 °C) PBS (1 × 106 cells/ml) with MitoTracker Green FM (MIG; Invitrogen; 200 nM), as described for ASCs staining. After a 30 min. incubation at 37 °C with agitation the cells were washed twice, counted and used for the analysis of mitochondria transfer from K562 to Tregs in indirect and direct cocultures in the same proportions as it was described for Treg-ASC cocultures. After 72 h the cells were collected and stained with anti-HLA-ABC antibodies (eBioscience, cat.56-9983-42) to separate Tregs (HLA-ABC−SSc°) from K562 cells (HLA-ABC−SSc°) during the analysis. Then data were collected by flow cytometry (LSRFortessa, BD Biosciences). In addition, images were taken using a confocal laser scanning microscope with 60× oil immersion objective lens. For each experiment stained and unstained Tregs and K562 cells were used as controls and analysed immediately after staining, as well as after 72 h. To verify whether HLA recognition plays a role in ASC-Treg mitochondria uptake, we performed a control experiment with a pan-specific anti-HLA class II antibody (IVA12 clone; BIOZOL Diagnostica Vertrieb GmbH, cat. CBT-104701) that was reported to block HLA class II molecules76. The antibody was added at a concentration of 10 μg/ml to ASCs cultures for 20 h before mitochondria transfer experiments to neutralize HLA class II antigens on ASCs. Then, ASCs were washed, stained with MIG and mixed with unstained Tregs.

HLA typing by next-generation sequencing (NGS) and analysis of HLA eplet mismatch load. HLA- A, B, C, DRB1 and DQ B1 were genotyped using a NGS method on Illumina platform (Illumina, San Diego, CA, USA). Sequencing-based HLA typing of the HLA genes A, B, C, DRB1 and DQB1 was carried out in 96-well
format within a semi-automated workflow by using MiAFora Flex5 typing kits (Immucor, Warren, NJ, USA). Long-range PCR amplification of five HLA loci was performed on Genomic DNA. Genomic DNA from Tregs and ASCs was extracted with the chemagic DNA CS200 Kit on Chemagic 360-D system (Wallac Oy, Mustionkatu 6, FI-20750 Turku, Finland). Results of sequencing were analyzed by MiAFora NGS software. Data were considered sufficient whenever the coverage reached 40 and the number of cNeats exceeded 50,000. The sequencing included the most extensive coverage of the HLA genome, especially with respect to five loci.

The immunogenicity of HLA antigens is determined by continuous and discontinuous short sequences of amino acids termed as functional epitopes or eplets. Based on this principle, an in silico approach—HLA-Matchmaker—was developed by Duquesney and colleagues. Currently, it is the only algorithm that serves for analysis of HLA eplet mismatch load (the number of donor-recipient eplet mismatches) which determines the immune response against allograft. In the current study, we used Version 3.1 of HLA-Matchmaker that is a freely available tool (http://www.epitopes.net/downloads.html). The repertoire of eplets analysed with HLA-Matchmaker derives from the International HLA Epitope Registry website (www.epiregistry.com) that is continually updated. Only experimentally antibody-verified eplets were taken into account to determine the HLA eplet mismatch load between Tregs and allogeneic ASCs in the co-cultures in the context of intercellular organelle transfer, cytokine secretion and impact on Treg function.

Analysis of cytokine secretion. Twenty-four and forty-eight hours after cell plating, SNs were collected from Tregs and ASC mononucleotides, as well as direct and indirect cocultures. SNs were frozen and stored at −80 °C until analysis. Concentrations of 48 mediators: sCD40L, EGF, eotaxin, PFG-2, FLT-3L, fractalkine, G-CSF, GM-CSF, GROα, IFNα2, IFNγ, IL-1α, IL-1β, IL-1RA (IL-1 receptor antagonist), IL-2, IL-3, IL-4, IL-5, IL-6, IL-7, IL-8; IL-9, IL-10, IL-12p70, IL-13, IL-15, IL-17A, IL-17E/IL-25, IL-17F, IL-18, IL-22, IFN-γ, IP-10, MCP-1/CCL2, MCP-3, M-CSF, MDC/CCL22, MIG/CXCL9 (monokine induced by IFNγ), MIP-1α/CCL3, MIP-1β, PDGF-AA, PDGF-AB/BB, RANTES/CCL5, TGFiα, TNFiα, TNFiβ and VEGF-A were measured with Human Cytokine/Chemokine/Growth Factor Panel A 48 Plex Kit (Merck) and analysed with Luminex (MAGPIX, Merck) at http://www.epitopes.net/downloads.html. The repertoire of eplets analysed with HLA-Matchmaker derives from the International HLA Epitope Registry website (www.epiregistry.com.br) that is continually updated. Only experimentally antibody-verified eplets were taken into account to determine the HLA eplet mismatch load between Tregs and allogeneic ASCs in the co-cultures in the context of intercellular organelle transfer, cytokine secretion and impact on Treg function.

References

1. Sakaguchi, S., Yamaguchi, T., Nomura, T. & Ono, M. Regulatory T cells and immune tolerance. Cell 133, 775–787 (2008).  
2. Gambineri, E. et al. Clinical, immunological, and molecular heterogeneity of 173 patients with the phenotype of immune dysregulation, polyendocrinopathy, enteropathy, X-linked (IPEX) syndrome. Front. Immunol. 9, 2411 (2018).  
3. Pilt, N. et al. Treg-mediated prolonged survival of skin allografts without immunosuppression. Proc. Natl Acad. Sci. USA 116, 13508–13518 (2019).  
4. Danby, R., et al. In vitro proportions of regulatory T cells in PBSC grafts predict improved survival after allogeneic haematopoietic SCT. Bone Marrow Transpl. 51, 110–118 (2016).  
5. Trzonkowski, P. et al. Hurdles in therapy with regulatory T cells. Sci. Transl. Med. 7, 304ps318 (2015).  
6. Fuchs, E. et al. Minimum information about T regulatory cells: a step toward reproducibility and standardization. Front. Immunol. 8, 1844 (2017).

ARTICLE NATURE COMMUNICATIONS | https://doi.org/10.1038/s41467-022-28338-0 | www.nature.com/naturecommunications
37. Vuerich, M., Harshes R. P., Robson, S. C. & Longhi, M. S. Dysregulation of adenosineergic signaling in systemic and organ-specific autoimmunity. Int. J. Mol. Sci. 20, 528 (2019).
38. Akkaya, B. et al. Regulatory T cells mediate specific suppression by depleting peptide-MHC class II from dendritic cells. Nat. Immunol. 20, 218–231 (2019).
39. Qureshi, O. S. et al. Trans-endocytosis of CD80 and CD86: a molecular basis for the cell-extrinsic function of CTLA-4. Sci. Rep. 3, 680–663 (2011).
40. Sansone, P. et al. Packaging and transfer of mitochondrial DNA via exosomes regulate escape from dormancy in hormonal therapy-resistant breast cancer. Proc. Natl Acad. Sci. USA 114, E9066–E9075 (2017).
41. Guan, B. C., Si, J. Q. & Jiang, Z. G. Blockade of gap junction coupling by glycyrhizic acids in guinea pig cochlear artery: a whole-cell voltage- and current-clamp study. Br. J. Pharm. 151, 1049–1060 (2007).
42. Han, H. et al. Bone marrow-derived mesenchymal stem cells rescue injured H9c2 cells via transferring intact mitochondria through tunneling nanotubes in an in vitro simulated ischemia/reperfusion model. Mol. Med. Rep. 13, 1517–1524 (2016).
43. Lisovsky, I., Istman, G., Bruneau, J. & Bernard, N. F. Functional analysis of NK cell subsets from NOD mice by 721.221 and K562 HLA-null cells. J. Leukoc. Biol. 97, 761–767 (2015).
44. Ryba, M. et al. Anti-TNF rescue CD4+Foxp3+ regulatory T cells in patients with type 1 diabetes from effects mediated by TNF. Cytokeine 55, 353–361 (2011).
45. Marek-Trzonkowska, N. et al. Therapy of type 1 diabetes with CD4+(+)/CD25(high)/CD127 regulatory T cells prolongs survival of pancreatic islets - results of one year follow-up. Clin. Immunol. 153, 23–30 (2014).
46. Marek, N., Mysiówka, J., Raczynska, K. & Trzonkowski, P. Membrane potential of CD4+(+) T cells is a subset specific feature that depends on the direct cell-to-cell contacts with monocytes. Hum. Immunol. 71, 666–675 (2010).
47. Trzonkowski, P. et al. CD4+CD25+ T regulatory cells inhibit cytotoxic activity of CD8+ and NK lymphocytes in the direct cell-to-cell interaction. Clin. Immunol. 112, 258–267 (2004).
48. Chen, J., Almo, S. C. & Wu, Y. General principles of binding between cell surface receptors and multi-specific ligands: a computational study. PLoS Comput. Biol. 13, e1005805 (2017).
49. Maurel, D. et al. Cell-surface protein-protein interaction analysis with time-course study. Proc. Natl Acad. Sci. USA 104, 337–342 (2007).
50. Khosravi, M. et al. Induction of CD4\(^+\) regulatory T cells by mesenchymal stem cells is associated with modulation of immune response. Cytokine Growth Factor Rev. 28, 306–314 (2015).
51. Couture, A. et al. HLA-class II antigen-presenting cells and HLA class II HLA mismatches determine risk of kidney transplant loss. Kidney Int. 87, 67–76 (2015).
52. Castello-Leon, E., Delleipane, S. & Fiorina, P. ATP and T-cell-mediated rejection. Curr. Opin. Organ Transpl. 23, 34–43 (2018).
53. Durand, M. et al. Increased degradation of ATP is driven by memory regulatory T cells in kidney transplantation tolerance. Kidney Int. 93, 1154–1164 (2018).
54. Tiercy, J. M. How to select the best available related or unrelated donor of hematopoietic stem cells? Haematologica 101, 680–687 (2016).
55. Williams, R. C., West, L. J. & Opelz, G. The risk of failure with HLA mismatch and recipient age in first pediatric (<18 years) kidney transplants. Transplant. Direct 4, e365 (2018).
56. Sapir-Pichhadze, R. et al. Epitopes as characterized by antibody-verified epitope mismatches determine risk of kidney transplant loss. Kidney Int. 97, 778–785 (2020).
57. Papichová, H. et al. The dual role of HLA-C in tolerance and immunity at the maternal-fetal interface. Front. Immunol. 10, 2730 (2019).
58. Couture, A. et al. HLA-class II artificial antigen presenting cells in CD4(+) T cell-based immunotherapy. Front. Immunol. 10, 1081 (2019).
59. Tilburgs, T. et al. Fetal-maternal HLA-C mismatch is associated with decidual T cell activation and induction of functional T regulatory cells. J. Reprod. Immunol. 82, 148–157 (2009).
60. Salvaney-Celades, M. et al. Three types of functional regulatory T cells control T cell responses at the human maternal-fetal interface. Cell Rep. 27, 2537–2547 (2019).
61. Beier, U. H. et al. Essential role of mitochondrial energy metabolism in Foxp3+ T-regulatory cell function and allograft survival. Faseb J. 29, 2315–2326 (2015).
62. Arous, N. et al. Extracellular ATP and CD39 activate cAMP-mediated mitochondrial stress response to promote cytarabine resistance in acute myeloid leukemia. Cancer Discov. 10, 1544–1565 (2020).
63. Zhao, J. & Perlman, S. Differential effects of IL-12 on Tregs and non-Treg T cell: effect of IL-7 and IL-25. PLoS ONE 7, e66241 (2012).
64. Zhao, L. et al. Interleukin-6 inhibits regulatory T cells and improves the proliferation and cytotoxic activity of cytokine-induced killer cells. J. Immunother. 35, 337–343 (2012).

Acknowledgements
The study was funded by National Science Centre, Poland (funding decision no. DEC-2011/01/D/NZ3/00262, granted to N.M.T.), project “International Centre for Cancer Vaccine Science” that is carried out within the International Research Agendas Programme of the Foundation for Polish Science co-financed by the European Union under the European Regional Development Fund (granted to T.H.), Polish Ministry of Science and Higher Education (grant no. IP2011 033771, granted to N.M.T.) and National Centre for Research and Development, Poland (grant no. STRATEGMED1/233681/1/NCBR/2014, granted to P.T.).

Author contributions
K.P., Z.U.W., M.K., I.P.M., A.S., J. Sakowska, J.H.S., H.Z., B.T., L.A. and N.M.T. performed experiments and contributed to data analysis. A.R. contributed to patient recruitment and sample collection. J. Siebert, E.S., P.T. and T.H. contributed to data analysis. N.M.T. conceived the study and was in charge of overall direction and planning of the study, designed experiments, contributed to data collection and interpretation and wrote the manuscript with input from all the authors. All authors edited and critically reviewed the paper and agree to the final version of the manuscript.

Competing interests
N.M.T. and K.P. are co-authors of two patent applications related to the presented content. N.M.T. and P.T. are shareholders of PolTREG S.A. company. The remaining authors declare no competing interests.

Additional information
Supplementary information The online version contains supplementary material available at https://doi.org/10.1038/s41467-022-28383-0.
Correspondence and requests for materials should be addressed to Natalia Maria Marek-Trzonkowska.
Peer review information Nature Communications thanks Sina Naserian and Lesley Smyth for their contribution to the peer review of this work. Peer reviewer reports are available.

Reprints and permission information is available at http://www.nature.com/reprints
Publisher’s note Springer Nature remains neutral with regard to jurisdictional claims in published maps and institutional affiliations.
