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Regulatory effects of microRNAs on monocytic HLA-DR surface expression

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Decreased monocytic HLA-DR expression is the most studied biomarker of immune competency in critically ill and autoimmune disease patients. However, the underlying regulatory mechanisms remain largely unknown. One probable HLA-DR dysregulation is through microRNAs. The aim of this study was to investigate the effects of specific microRNAs on HLA-DR expression in human monocytic cells. Four up- and four down-HLA-DR-regulating microRNAs were identified, with hsa-miR-let-7f-2-3p showing the most significant upregulation and hsa-miR-567 and hsa-miR-3972 downregulation. Anti-inflammatory glucocorticoid medication Dexamethasone-decreased HLA-DR was significantly restored by hsa-miR-let-7f-2-3p and hsa-miR-5693. Contrarily, proinflammatory cytokines IFN-γ and TNF-α-increased HLA-DR were significantly reversed by hsa-miR-567. Clinically, paired plasma samples from patients before and one day after cardiac surgery revealed up-regulated expression of hsa-miR-5693, hsa-miR-567, and hsa-miR-3972, following the major surgical trauma. In silico approaches were applied for functional microRNA-mRNA interaction prediction and candidate target genes were confirmed by *q*PCR analysis. In conclusion, novel monocytic HLA-DR microRNA modulators were identified and validated in vitro. Moreover, both the interaction between the microRNAs and anti- and proinflammatory molecules and the up-regulated microRNAs identified in cardiac surgery highlight the potential clinical relevance of our findings.

Keywords: Cytokine · Human leukocyte antigen (HLA) · Immunosuppression · MHC class II · microRNA (miRNA) · Monocyte

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

Introduction

Decreased monocytic Human Leukocyte Antigen–DR isotype (HLA-DR) surface expression has hitherto been the most studied

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biomarker of systemic inflammatory response syndrome (SIRS), such as sepsis, and is linked to a higher risk of acquiring severe nosocomial infections, and higher mortality in sepsis [1]. HLA-DR belongs to the MHC-II family, which is essential for accurate and efficient immune responses. MHC-II expression regulation is mainly at the transcriptional level, for example, MHC-II transactivator CIITA activates *HLA* promotors, and *CIITA* mutations can lead to severe immunodeficiency such as bare lymphocyte syndrome [2]. Other MHC-II regulators include cytokines, pH values, kinases, and TLR signaling [3].

Until now, few underlying regulatory mechanisms of HLA-DR expression have been identified. One probable form of regulation is through microRNAs (miRNAs) [4]. Reduced HLA-DR levels on APCs, such as monocytes, have been associated with autoimmune diseases, critical immunosuppression, severe secondary bacterial infections, and end-organ damage in critical illness, such as the severe cases of coronavirus disease 2019 [5–8]. The perioperative monocytic HLA-DR measurement has been postulated as a potential biomarker of sepsis [9, 10]; however, earlier detection is needed, especially among patients at a higher risk of SIRS who would benefit from receiving in-time immunostimulants [8].

In recent years, miRNA's clinical applications have gained increasing recognition, particularly in cancer medicine [11, 12]. In perioperative medicine, on the other hand, differential expression of circulating miRNAs, extracellular or intracellular, is emerging as a novel diagnostic approach to identifying and treating sepsis in SIRS patients [13–16]. Nevertheless, we are still awaiting empirical evidence that can link MHC-II dysregulation to associated miRNAs [17–19].

A recent flow cytometry-based high-throughput RNAi screening identified miRNAs either up- or down-regulating HLA-DR surface expression in an APC-like human melanoma cell line, MelJuSo [4]. Although MelJuSo has stable constitutive HLA-DR surface expression and provides an excellent in vitro platform for conducting such RNAi screening for HLA-DR regulators [3, 4], the translatability of MelJuSo study outcomes in clinical immunology is limited, due to its lack of immune-specific cell properties. Hence, in the current study, we employed a human leukemia monocytic cell line, THP-1, to investigate miRNA's HLA-DR regulation and compare results to MelJuSo studies. The aim was to identify and validate miRNA regulators for monocytic HLA-DR surface expression.

Our results identified four up- and four downregulating miRNAs for monocytic HLA-DR expression. miRNA co-regulatory effects of either anti-inflammatory glucocorticoid medication or proinflammatory cytokines were confirmed, further supporting the physiological and clinical relevance of selected miRNA effectors. Plasma miRNA analyses were conducted in patients before and one day after undergoing surgery with cardiopulmonary bypass which revealed, intriguingly, three up-regulated miRNAs upon major surgical trauma, suggesting their clinical potential as immunosuppression biomarkers. Last, in silico approaches were applied for functional miRNA-mRNA interaction prediction to identify candidate target mRNAs of the selected miRNA effectors, which was confirmed by subsequent quantitative real-time PCR (qPCR) analyses.

Results

Monocytic HLA-DR surface expression regulated by miRNAs

In a recent flow cytometry (FCM)-based high-throughput miRNA screening of human miRNA compounds stored in the miRIDIAN miRNA mimic library (19.0, CS-001030, Dharmacon), about 2000 miRNAs were examined for their HLA-DR-regulating effects in a human APC-like melanoma cell line, MelJuSo [4]. Based on the calculated rank order of the RNAi screening, we further selected and pre-tested 20+ among the top-ranked miRNAs for their potential HLA-DR up- and downregulating effects in different human monocytic cell lines and were able to choose human monocytic THP-1 cells and optimize the miRNA transfection for the follow-up experiments. After the pre-tests (Supporting information Fig. S1), we further narrowed down the candidate pool to eight miRNA regulators for HLA-DR expression. In the next transfection series, each miRNA transfection was repeated 8 times within experiments, and the surface HLA-DR expression was analyzed by FCM, computed as median fluorescence intensity (Fig. 1A). All experiments were replicated three times with three different THP-1 cell lines. Despite different statistical significance due to high variation among experiments and cell lines, results from all experiments generally revealed a similar HLA-DR upregulation by miR-let-7f-2-3p, miR-5693, miR-500a-3p, and miR-15a-5p and downregulation by miR-3971, miR-193-3p, miR-567, and miR-185-5p, compared with the miR-cel67 control (Fig 1B–D). THP-1 cell viability of the transfection assay was estimated to be 98% using a fixable viability dye eFluor 506 (eBioscience) (Supporting information Fig. S2). Moreover, miRNA delivery efficiency was estimated to be 74%, using a Dy547-labelled miRNA hairpin inhibitor transfection control (Supporting information Fig. S3).

To compare the miRNA effect in MelJuSo cells, we conducted the same transfection experiment, also in three replications. Interestingly, not miR-let-7f-2-3p but miR-15a-5p displayed the strongest effect on HLA-DR upregulation. Furthermore, two down-regulators for THP-1 — miR-185-5p and miR-193-3p — led to HLA-DR upregulation in MelJuSo (Supporting information Fig. S4). This discrepancy might reflect distinct underlying regulatory pathways of APCs versus nonimmune cells that are poor in immune regulatory molecules, for example, in TLRs (data not shown), which are known to interact with antisense and siRNA [3, 20, 21].

Next, to validate miRNA-regulating effects at the *HLA-DRA* transcript level [22], we collected THP-1 cells 48 h posttransfection and conducted qPCR analysis. In general, in THP-1 cells the four up- and four down-HLA-DR miRNA modulators resulted in gene-regulating effects similar to those of protein expression (Supporting information Fig. S5B vs. Fig. 1). *HLA-DRB1* and *CIITA* expression, on the other hand, were not or were only

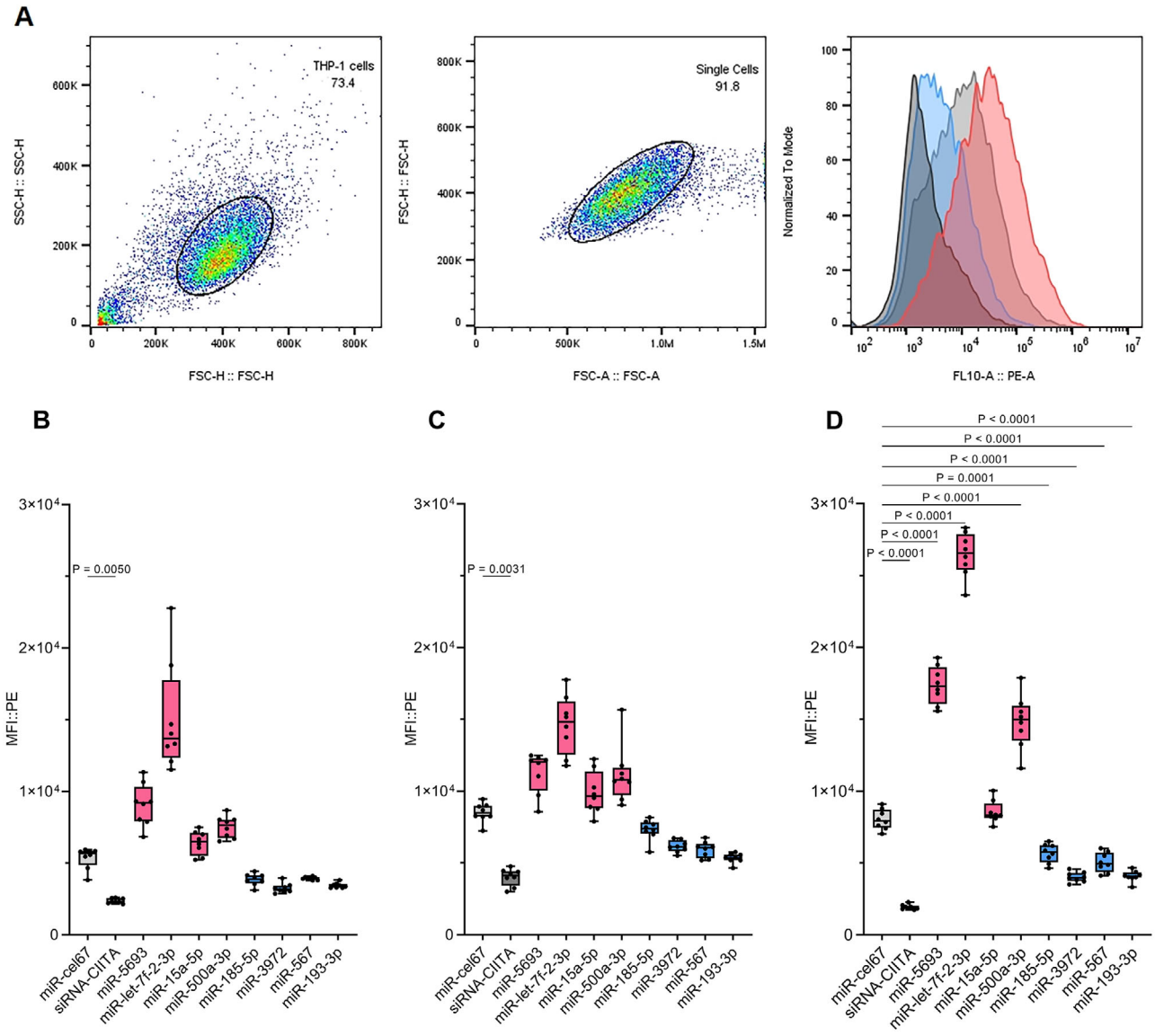

Figure 1. Regulating effect of eight selected miRNAs on HLA-DR surface expression. Flow cytometry was conducted to estimate HLA-DR surface expression 72 h posttransfection in human cell line THP-1. (A) Representative dot plots gating THP-1 population, single cells, and histogram of surface HLA-DR protein expression levels in gated THP-1 single cells transfected with different small noncoding RNA molecules (light grey: negative control miRNA mimics targeting miR-cel67, dark grey: positive control siRNA targeting CIITA, blue: downregulating miRNAs, and red: upregulating miRNAs). (B–D) Box and whisker plots describe three independent experimental replicates with different THP-1 cell lines. The center line denotes the median value (50th percentile), while the box contains the 25th to 75th percentiles of the dataset. The whiskers mark the minimum and maximum values, and all data points are marked with dots ($N = 8$). Comparisons of the median fluorescence intensity (MFI) of surface HLA-DR among various treatment groups (same color codes as in A) to the miR-cel67 group were made using either Kruskal–Wallis test and post hoc analysis with Dunn's multiple comparison test (B, C) or one-way ANOVA and Durnett's multiple comparison test, with a single pooled variance (D). Treatment significance ($p < 0.0001$) was found in all three experimental replications. For post hoc multiple comparisons, only p-values less than or equal to 0.05 are displayed in the figure.

marginally regulated (Supporting information Fig. S5C, A). Comparable results were found in MelJuSo cells (Supporting information Fig. S6A–C) [4].

Since single-miRNA treatments could not easily trigger significant regulating effects, we further verified the HLA-DR regulation using a double-transfection experiment, where we conducted two consecutive miRNA transfections with a 6 h time interval between them. All miRNA combinations were tested, including the same miRNA twice as a comparison (Fig. 2). Overall, the result not only confirmed both additive (i.e. two upregulators) and deductive (i.e. one up- and one downregulator) HLA-DR-regulation effects. Moreover, it beautifully reflected the different degree of regulation during single transfections (e.g., HLA-DR of "miR-5693+miR-let-7f-2-3p" > "miR-5693+miR-5639" > "miR-5693+miR-500a-3p" > "miR-5693+miR-15a-5p" in Fig. 2 versus miR-let-7f-2-3p > miR-5693 > miR-500a-3p > miR-15a-

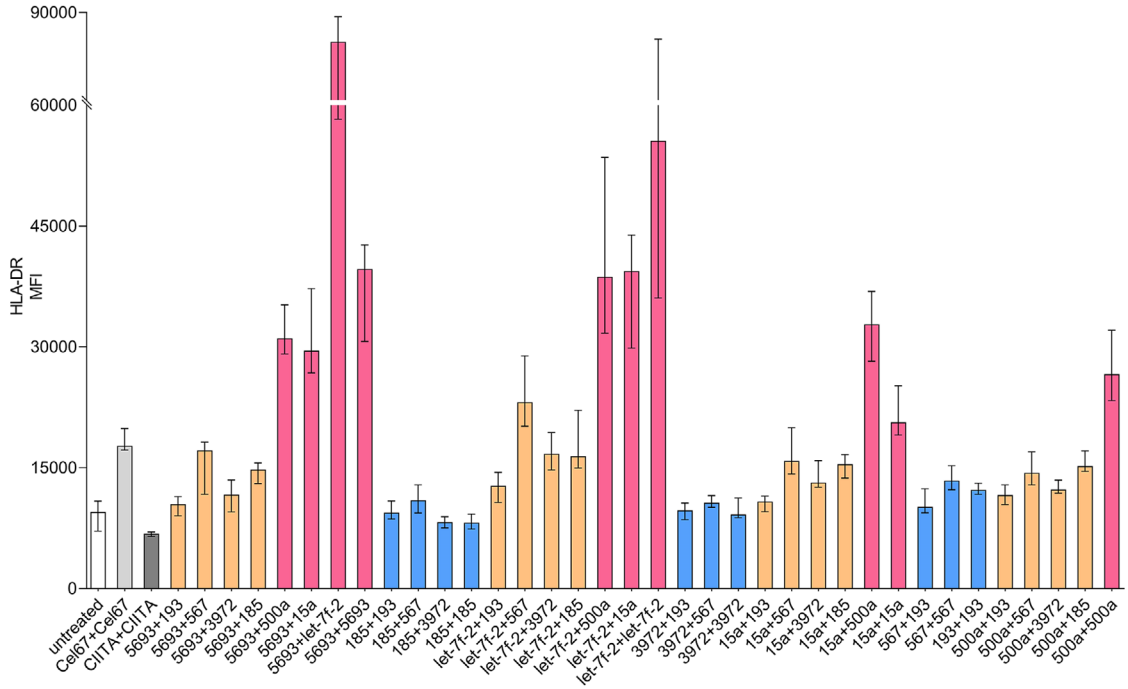

Figure 2. Regulating effect of double-transfection of selected miRNAs on HLA-DR surface expression. Two consecutive miRNA transfections with a 6 h time interval in between were conducted with all miRNA-combinations, including twice the same miRNAs, negative controls (plain medium or miR-cel67), and positive controls (siRNA-CIITA), yielding a total of 39 treatment groups (white: untreated control; light grey: twice negative control; dark grey: twice positive control, blue: twice down-regulating miRNAs, red: twice up-regulating miRNAs, and orange: once up- and once downregulating miRNAs). Flow cytometry was conducted to estimate HLA-DR surface expression 72 h posttransfection in human cell line THP-1. The bar figure represents the complete dataset ($N = 8$ for all treatment groups). The height of the bar marks the computed median, and the error bar shows the interquartile range.

5p in Fig. 1). Thus, in THP-1 cells, both HLA-DR gene and surface protein expression can be up- and downregulated by specific miRNA modulators, alone or in combination.

miRNAs can modulate the monocytic HLA-DR regulation induced by pro- and anti-inflammatory molecules

Both HLA-DR low expression and various pro- and anti-inflammatory cytokines' overproduction have been linked to severe infections and sepsis [23, 24]. Moreover, HLA-DR downregulation during systemic inflammation and septic shock was largely mediated by IL10 and glucocorticoids [25, 26]. We studied whether exogenous miRNAs could modulate HLA-DR dysregulation induced by inflammatory molecules. We first stimulated THP-1 cells with the proinflammatory cytokines IFN-γ and TNF-α, both known to increase the baseline HLA-DR expression in THP-1 [27], or conversely with the glucocorticoid dexamethasone, which decreases HLA-DR expression [26]. Cells were transfected with individual miRNAs after treatment. FCM analyses of surface HLA-DR expression at 48 h posttransfection revealed that IFN-γ stimulation largely increased baseline HLA-DR surface expression compared with untreated cells and that CIITA_siRNA and miR-567 significantly reduced HLA-DR expression (Fig. 3A). Surprisingly, one down-regulator — miR-185 — further increased the HLA-DR expression. Like IFN-γ treatment, HLA-DR expression was upregulated by IFN-γ + TNF-α stimulation, and CIITA_siRNA and miR-567 again evoked counter-regulating effects; moreover, miR-5693 and miR-let-7f-2-3p further increased HLA-DR expression significantly (Fig. 3B). Last, dexamethasone treatment decreased baseline HLA-DR expression and both miR-5693 and miR-let-7f-2-3p significantly restored HLA-DR expression (Fig. 3C), suggesting their potential to improve dexamethasone-induced immunosuppression [28]. Our results provide the first evidence that miRNAs can interact with pro- and anti-inflammatory molecules that regulate MHC-II molecules.

Novel clinical evidence of upregulated miR-3972, miR-567, and miR-5693 following a major surgical trauma

Based on the in vitro evidence of monocytic HLA-DR regulatory effects of selected miRNAs, we postulated that they may be involved in perioperative immunosuppression that is directly linked to monocytic HLA-DR downregulation. To test this hypothesis, we analyzed the plasma miRNAs in twenty-one patients undergoing surgery with cardiopulmonary bypass and compared miRNA expression before and 24 h after surgery. All eight targets plus two endogenous miRNAs in plasma were quantified, with the results revealing upregulation of three miRNAs — miR-

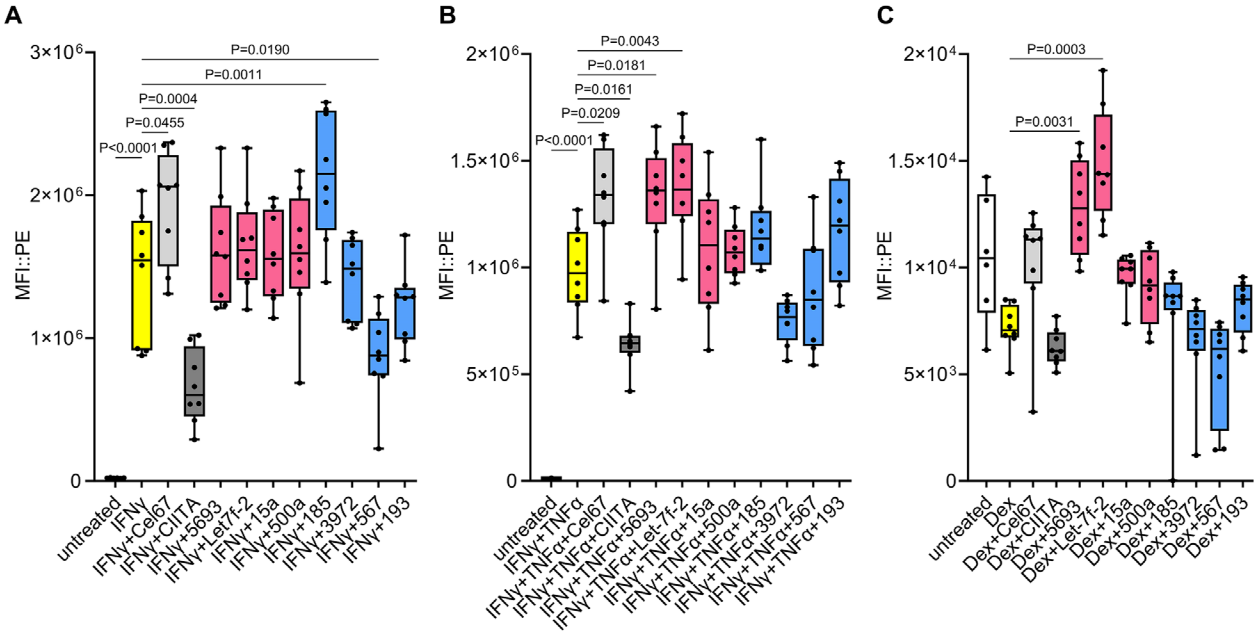

Figure 3. Co-regulating effect of pro-/anti-inflammatory molecules and eight selected miRNAs on HLA-DR surface expression. Flow cytometry was conducted to estimate HLA-DR surface expression 48 h posttreatment in human cell line THP-1. (A–C) Box and whisker plots describe the dataset of median fluorescence intensity (MFI) of surface HLA-DR after miRNA transfection under treatments of (A) IFN-γ (10 ng/mL), (B) IFN-γ and TNF-α (each 10 ng/mL), and (C) dexamethasone (100 ng/mL). The center line denotes the median value (50th percentile), while the box contains the 25th to 75th percentiles of the dataset. The whiskers mark the minimum and maximum values, and all data points are marked with dots (N = 8). Comparisons of the surface HLA-DR MFI among various treatment groups (white: untreated, yellow: drug treatment without transfection, light grey: drug treatment with transfection negative control of miR-cel67, dark grey: drug treatment with transfection positive control of siRNA-CIITA, blue: drug treatment with down-regulating miRNAs, and red: up-regulating miRNAs) to the drug-treatment alone were done using either one-way ANOVA and Holm–Šídák's (A) or Dunnett's (B) multiple comparisons tests assuming equal SDs or Kruskal–Wallis test and Dunn's multiple comparisons test (C). Treatment significance ($p < 0.0001$) was found in all three experimental replications. For post hoc multiple comparisons, only p-values less than or equal to 0.05 are displayed in the figure.

3972, miR-567, and miR-5693 — upon major surgery (Fig. 4, Supporting information Table S1 and Fig. S7). Like other cell-free samples, plasma normally contained low amounts of extracellular miRNAs. These were further diluted during perioperative fluid administration, leading to a generally higher Crossing point (Cp) calculation postsurgery, as seen with both endogenous miRNAs (Fig. 4 and Supporting information Table S1). Intriguingly, the three miRNAs were mostly undetected before surgery (i.e. Cp value higher than the detection threshold of 40 in LightCycler 480), however, revealed lower Cp values postsurgery (i.e. higher expression, Fig. 4 and Supporting information Table S1). Thus, they may serve as potential miRNA biomarkers for perioperative immunosuppression related to HLA-DR downregulation.

Candidate binding targets of HLA-DR-regulating miRNAs

To identify potential mRNA binding targets of selected miRNAs, we adopted in silico approaches using the TargetScan database for target prediction [29–31]. The search results predict the interaction of each individual miRNA with various candidate genes (Supporting information Table S2). We then conducted a literature search to narrow down the candidate pool to immune-related genes and conducted qPCR pre-analyses (Supporting information Table S2 and Fig. S8). Based on pre-tests, seven candidate genes were identified for different miRNAs. Single miRNA transfections in both MelJuSo and THP-1 cells were conducted prior to qPCR analyses of corresponding target genes. The predicted interactions were: miR-15a-5p::*BTLA*, miR-15a-5p::*SMPD*, miR-185-5p::*RAB35*, miR-500a::*SOCS*, miR-let-7-2-3p::*HVCN1*, miR-let-7-2-3p::*TCF7L2*, and miR-185-5p/miR-193-3p::*KLF7*. The results revealed that gene regulations were generally more pronounced in MelJuSo cells (Fig. 5A) compared with THP-1 (Fig. 5B). This might be partially explained by the considerably higher transfection efficiency in MelJuSo cells. Note that the mRNA regulation effects of *SMPD*, *SOCS*, and *TCF7L2* were only observed in MelJuSo.

Discussion

Four up- and four downregulating miRNAs for monocytic HLA-DR surface expression were identified by FCM and qPCR. miRNA co-regulatory effects of anti-inflammatory glucocorticoid and pro-inflammatory cytokines were confirmed in vitro. Clinically, three miRNAs revealed an increased plasma level following a major surgery. In silico methods helped identify seven candidate target genes for miRNA regulators, and their interactions were verified by miRNA transfection and qPCR analysis.

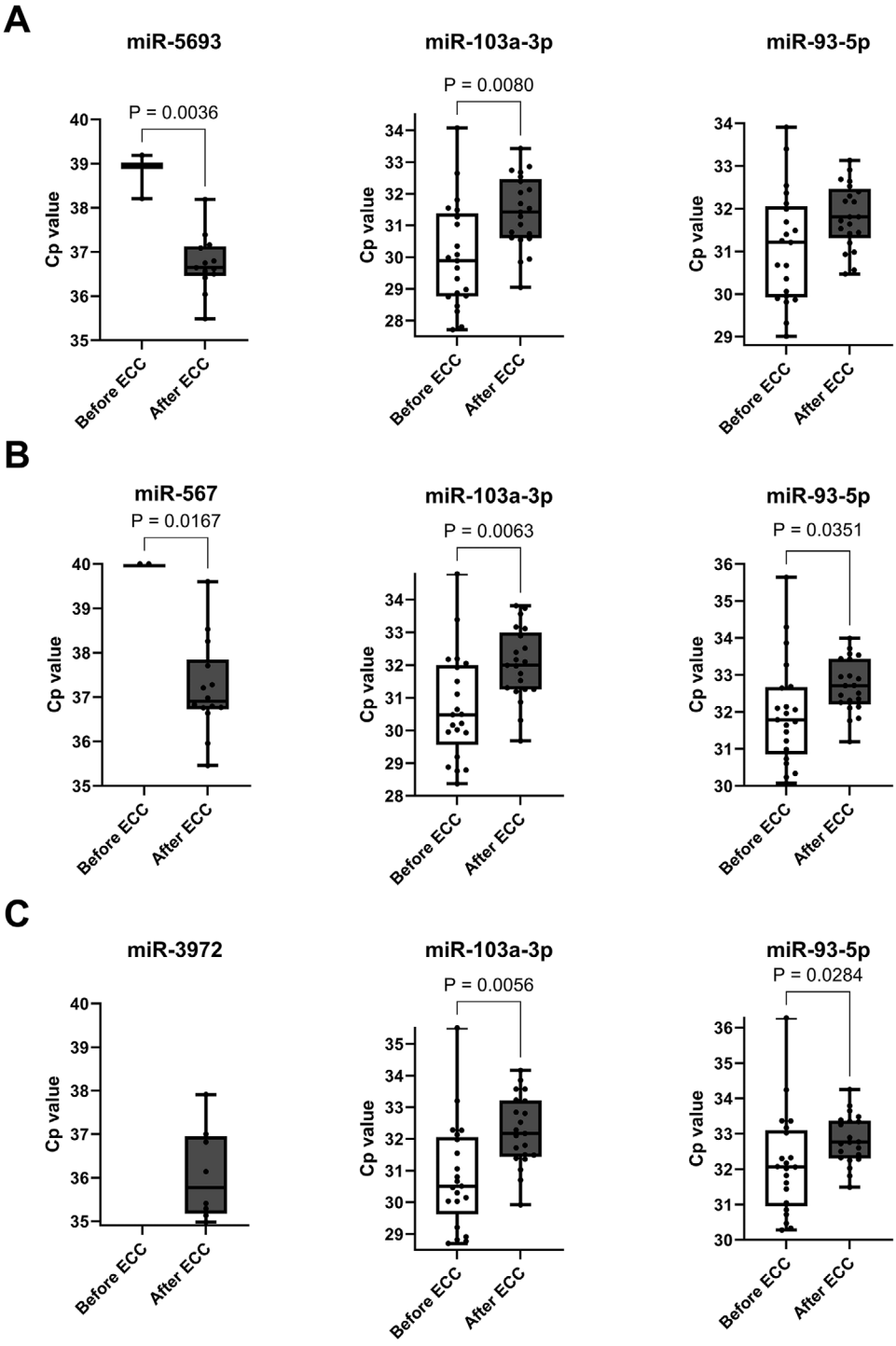

Figure 4. Upregulations of three extracellular plasma miRNAs upon major surgical trauma. Real-time quantitative PCR (qPCR) was performed to estimate extracellular miRNA expression in plasma samples of 21 patients before and 24 h after cardiac surgery with cardiopulmonary bypass. All PCR reactions were done in duplicate. Box and whisker plots describe the mean qPCR crossing point (Cp) values for all detected samples before (before ECC) and 24 h after (after ECC) the cardiac surgery. The center line denotes the median value (50th percentile), while the box contains the 25th to 75th percentiles of the dataset. The whiskers mark the minimum and maximum values, and all data points are marked with dots. Undetected samples with Cp value beyond the detection threshold of 40 were excluded. For all three target miRNAs has-miR-5693 (A), has-miR-567 (B), and has-miR-3972 (C), the endogenous plasma miRNAs miR-103a-3p and miR-93-5p were quantified alongside for validation and comparison. For miR-103a-3p and miR-93-5p, Cp values before and after ECC were compared using the Wilcoxon matched-pairs signed rank test, as for miR-5693 and miR-567, Mann–Whitney test, and the exact *p*-values were computed, which considered ties among values. Significance ($p < 0.005$) was found in all comparisons.

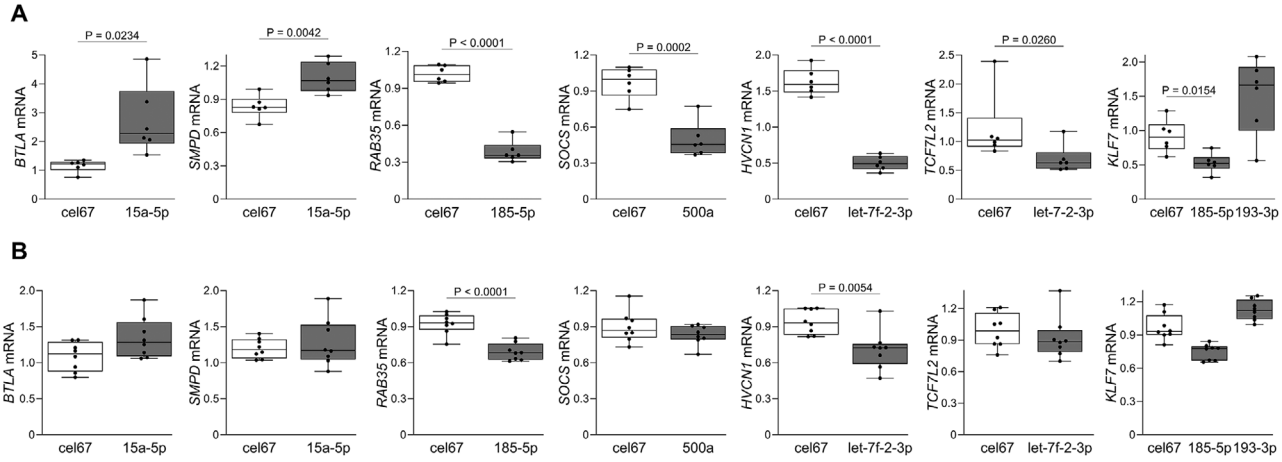

Figure 5. Regulating effect of single selected miRNAs on their predicted target genes. Real-time quantitative PCR (qPCR) was performed to estimate mRNA transcript expression 48 h posttransfection in human cell lines (A) MelJuSo and (B) THP-1. Two housekeeping genes glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and hypoxanthine phosphoribosyl-transferase 1 (HPRT1) were used as reference genes. The relative quantification is calibrator-normalized with efficiency correction and the gene expression results are presented as a normalized ratio. Seven predicted interactions include miR-15a-5p::*BTLA*, miR-15a-5p::*SMPD*, miR-185-5p::*RAB35*, miR-500a::*SOCS*, miR-let-7-2-3p::*HVCN1*, miR-let-7-2-3p::*TCF7L2*, and miR-185-5p/miR-193-3p::*KLF7*. Box and whisker plots describe the resulting gene regulations by corresponding miRNA transfections (white: negative control miR-cel67, grey: selected miRNA). The center line denotes the median value (50th percentile), while the box contains the 25th to 75th percentiles of the dataset. The whiskers mark the minimum and maximum values, and all data points are marked with dots (N = 8). (A) In MelJuSo cells, comparisons of the normalized ratio of each miRNA treatment to the miR-cel67 group were made using an unpaired t-test with Welch's correction, except for miR-let-7-2-3p::*TCF7L2* using Kolmogorov–Smirnov test and miR-185-5p/miR-193-3p::*KLF7* using Brown–Forsythe and Welch ANOVA tests for post hoc multiple comparisons. (B) In THP-1 cells, comparisons of the normalized ratio of each miRNA treatment to the miR-cel67 group were made using an unpaired *t*-test with Welch's correction, except for miR-185-5p/miR-193-3p::*KLF7* using Brown–Forsythe and Welch ANOVA tests for post hoc multiple comparisons. Only *p*-values less than or equal to 0.05 are displayed in the figure.

Specific miRNAs exert distinct HLA-DR regulatory effects in different human cell lines

Previous in vitro studies of HLA-DR regulation by siRNAs or miRNAs were mainly conducted in non-APC human MelJuSo cells. These possess the advantages of both stable constitutive HLA-DR surface expression and high transfection efficiency [3, 4]. In the current study, we performed miRNA transfection assays in human monocytic THP-1 cells for HLA-DR regulation for the first time. Despite comparable experimental paradigms, candidate miRNAs could lead to different HLA-DR-regulating effects (e.g. both miR-185-5p and miR-193-3p upregulated HLA-DR in MelJuSo but down-regulated HLA-DR in THP-1 cells; Fig 1 vs. Fig. S4). While MelJuSo lacks many immune-specific functions, specific miRNAs that exert distinct HLA-DR regulations in THP-1 may suggest the underlying MHC-II/HLA-DR regulation involving TLR or other major immune factor signaling pathways.

miRNAs interact with anti-inflammatory glucocorticoids and proinflammatory cytokines for HLA-DR regulation

Abundant *in vitro* and clinical evidence has linked cytokine production to HLA-DR expression in critical inflammation states [23, 32, 33]. Clinically, glucocorticoids (e.g. cortisol) [26, 34] and catecholamines (e.g. norepinephrine) [35, 36] downregulate HLA-DR, which could cause further immunosuppression. Our data not only support the interaction between miRNAs and glucocorticoids or cytokines, the counteraction of HLA-DR regulation by miRNAs, for example, miR-let-7f-2-3p and miR-5693, restored the glucocorticoid-induced HLA-DR downregulation, further suggesting miRNAs could serve as potential co-treatment options for stabilizing immune responses.

Plasma miR-3972, miR-567, and miR-5693 as potential biomarkers for perioperative immunosuppression

Major surgical trauma often results in immunosuppression, with hallmarks of monocytic deactivation and Th1/Th2 cytokine imbalance [37, 38]. Likewise, monocytic HLA-DR downregulation has been the most reliable biomarker for evaluating immunosuppression in sepsis. In this study, we successfully identified three novel miRNAs showing markedly increased secretion in patients' plasma following major surgery. Since these miRNAs were proven to modulate monocytic HLA-DR expression, further investigation of their immune regulatory effects will help us understand underlying HLA-DR regulatory mechanisms, both in physiological and critical immune conditions.

Candidate miRNA target genes for MHC-II regulation

Both in silico prediction of miRNA binding targets and literature review allowed us to select top candidate genes for HLA-DR regulation, which was further confirmed by miRNA transfection/qPCR. Among the identified target genes, *RAB35*, encoding a

small GTPase RAB35, is associated with a peptide-loaded MHC-II internalization and recycling process [39]. Thus, our new findings that miR-185-5p downregulated both *RAB35* and HLA-DR surface expression, are consistent with the proposed RAB35 function about MHC-II regulation. Another gene–*HVCN1*–, encoding the proton channel which expresses exclusively in immune cells [40], was downregulated by miR-let-7-2-3p — an HLA-DR upregulator. Until now we have had a limited understanding of HCVN1's role in antigen presentation. However, its expression in bone marrow-derived dendritic cells (DCs) has been confirmed in mice [41]. HCVN1 could regulate antigen presentation and recognition in DCs and B cells through their role in ROS production [40–42]. Our research may contribute to a further understanding of proton channels' roles in regulating innate and adaptive immune responses.

Our study has several limitations. First, most data were generated using human MelJuSo and THP-1 cell lines. While both have proven to be excellent in vitro platforms for conducting such an RNAi study, owing to their stable constitutive HLA-DR expression and sufficient transfection efficiency, the translatability of the study outcomes to clinical immunology is limited and will rely on comparable experimental findings using human primary cells. Although our preliminary data from freshly isolated primary human monocytes and as well the monocyte population in human PBMC culture have confirmed similar miRNA-regulating trends of monocytic HLA-DR surface expression (Fig. S9), a stable ex vivo whole-blood miRNA transfection system will not only more closely resemble the physiological conditions but provide an optimal platform for the follow-up mechanistic study of specific miRNA regulatory pathways. Second, individual miRNA candidates identified through the study may exert their HLA-DR regulatory with different time courses. Thus, to study specific miRNA-regulating effects on HLA-DR regulation, individual miRNAs should be studied in the context of their gene regulation dynamics. Finally, although our clinical study was limited to a small patient number, the within-subject comparison before and after surgery has delivered promising preliminary results of novel miRNA biomarkers for surgical trauma-induced immunosuppression. Follow-up studies including a bigger cohort size and outcomes from different cohorts (e.g. sepsis/SIRS patients versus cardiac surgery patients) will be indispensable for confirming and identifying novel miRNA biomarkers of dysregulated immune responses in critical medicine.

In summary, we identified novel miRNA modulators for monocytic HLA-DR regulation. The potential clinical relevance of our study outcomes is further supported by HLA-DR co-regulatory effects, with various clinical immune modulators. The upregulated plasma miRNAs identified in cardiac surgery patients strongly suggest the potential usage of these molecules as immunosuppression biomarkers. Our results promote the study of miRNA-mediated HLA-DR regulation under both physiological and critical immune conditions and shall pave the way for future intensive-care medicine applications, including therapeutic targeting.

Materials and methods

Cell culture, miRNA transfection, and flow cytometry

Human monocytic THP-1 cells were cultured in RPMI 1640 medium supplemented with 10% FBS, 200 mM glutamine, 100 units/mL penicillin, and 10 μg/mL streptomycin. Human melanoma MelJuSo cells were cultured in Iscove's modified Dulbecco's medium with 10% FBS, 200 mM glutamine, 100 units/mL penicillin, and 10 μg/mL streptomycin. All chemicals were purchased from Sigma-Aldrich. The siRNA against CIITA, siRNA Hs_CIITA_2 (Qiagen), was used as a transfection-positive control, and the miRIDIAN microRNA mimic, cel-miR-67, as a negative control. Moreover, a Dy547-labeled miRNA hairpin inhibitor based on cel-miR-67 was used to monitor miRNA delivery. All miRIDIAN miRNA mimics were purchased from Dharmacon and Merck.

THP-1 cells were transfected using RNAiMAX (Thermo Fisher) with up to 5×10^4 cells/well in 96-well plates. MelJuSo cells used DharmaFECT transfection reagent #1 (Dharmacon), with up to 60–70% confluency in single wells. Generally, transfections were performed according to the manufacturers' advice. After transfection, cells were incubated at 37°C in 5% CO_2, for up to 72 h depending on the follow-up experiments. For antibody staining before FCM analysis, surface HLA-DR was labeled with a PE anti-human-HLA-DR antibody (BioLegend). Either CytoFLEX (Beckman, for Fig. 1) or AURORA (Cytec, for Figs 2 and 3, Fig. S4) was used for FCM. Acquired FCM data were analyzed with FlowJo software v_10.8.1 (FlowJo, LLC).

Titration pretests were performed for all selected compounds to determine the final concentrations used for co-stimulation experiments. Cells were treated with 100 ng/mL dexamethasone (Mepha) or 10 ng/mL IFN-γ and TNF-α (BioLegend), followed by miRNA transfections. FCM analyses were carried out 48- and 72-h posttransfection.

Quantitative real-time PCR

To validate the gene regulation effects of miRNA transfections on *HLA-DRA* and other candidate target genes, qPCR analyses were performed 48 h posttransfection. Briefly, total RNA was extracted using a HighPure RNA Extraction Kit (Roche). RNA quantity and quality were verified by NanoDrop2000 spectrophotometer (Thermo Fisher). cDNA was synthesized using GoScript Reverse Transcriptase (Promega). *qPCR* was performed in LightCycler 480 (Roche) using TaqMan Fast Advanced Master Mix and TaqMan Gene Expression Assay (Thermo Fisher) targeting *HLA-DRA, HLA-DRB1, CIITA, BTLA*, *SMPD*, *RAB35*, *SOCS*, *HVCN1*, *TCF7L2*, and *KLF7*, along with housekeeping genes *GAPDH* and *HPRT1*. Identical cDNAs from THP-1 cells or MelJuSo cells were used throughout *qPCR* assays as calibrators. Standard curves of all genes were generated independently. Duplicates were made for each

PCR reaction. Data were analyzed using the "Advanced Relative Quantification Module" of LightCycler 480 software version 1.5.0.

Participants' blood collection and plasma miRNA analysis

The study participants were patients undergoing surgery with cardiopulmonary bypass. Twenty-one patients were included in our pilot study, which was approved by the local ethical committee (KEK Bern, 2018-01272). All patients signed written informed consent. Inclusion criteria were written informed consent, age $>$ 18 years, and nonemergency surgery. Exclusion criteria were no written informed consent or patients who retrospectively expressed their wish to be excluded.

EDTA blood was collected at induction of anesthesia (0 h) and 24 h postinduction. Plasma samples were stored at −80° for further analysis. miRNeasy Serum/Plasma Advanced Kit was used for RNA extraction, and a miRCURY LNA RT kit was used for cDNA synthesis (Qiagen). Different miRCURY LNA RNA Spike-in kits were used during RNA extraction or cDNA synthesis for quality controls: UniSp2/4/5 for RNA isolation efficiency and UniSp6 for both RT and PCR efficiency. Additionally, miRCURY LNA miRNA Probe PCR Assays has-miR-451a and hsa-miR-23a-3p were applied as hemolysis controls to exclude potential sample contamination with lysed red cells. Only samples passing all PCR quality controls were further analyzed using miRCURY LNA miRNA Probe PCR Assays of miRNAs of interest: has-miR-let-7f-2-3p, has-miR-5693, has-miR-500a-3p, has-miR-15a-5p, has-miR-3971, has-miR-193-3p, has-miR-567, and has-miR-185-5p. Two endogenous miRNAs, hsa-miR-103a-3p and has-miR-93-5p, were used as reference genes. miRNA qPCR was performed in LightCycler 480 and acquired data were processed and analyzed by performing the "Absolute Quantification Analysis" using the "Second Derivative Maximum method" provided by the LightCycler 480 system software version 1.5.0.

In-silico miRNA–mRNA interaction prediction

To predict mRNA binding targets of selected miRNAs, we applied in silico approaches using TargetScan Human 8.0. database and double-checked with miRDB [29–31]. The search results predict the interaction of individual miRNAs with various candidate genes, each with a cumulative context score. For hits scores higher than 0.4, a subsequent literature search was conducted with the aim of targeting immune-related candidate genes for subsequent qPCR analysis.

Statistical analysis

Statistical analyses were performed using either ordinary one-way ANOVA test and post hoc Dunnett's or Holm-Šídák's multiple comparison tests — when data were normally distributed — or otherwise Kruskal–Wallis test, followed by Dunn's multiple comparison for post hoc analysis, or Mann–Whitney test. Data are presented either as box plots containing the interquartile range, whiskers showing minimum to maximum, and all data points displayed as round dots; or bar plots with the height of the bar representing median and error bars showing interquartile range. GraphPad Prism software version 9.5.0 (730) was used for the statistical analysis and graphing of the results.

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Data availability statement: The data that support the findings of this study are available from the corresponding author upon reasonable request.

References

1 **Sebastian, A., Sanju, S., Jain, P., Priya, V. V., Varma, P. K. and Mony, U.**, Non-classical monocytes and its potential in diagnosing sepsis post cardiac surgery. *Int. Immunopharmacol.* 2021. **99**: 108037.

2 **Chang, C. H., Gourley, T. S. and Sisk, T. J.**, Function and regulation of class II transactivator in the immune system. *Immunol. Res.* 2002. **25**: 131–142.

3 **Paul, P., van den Hoorn, T., Jongsma, M. L., Bakker, M. J., Hengeveld, R., Janssen, L., Cresswell, P. et al.**, A genome-wide multidimensional RNAi

screen reveals pathways controlling MHC class II antigen presentation. *Cell* 2011. **145**: 268–283.

4 **Houseman, M., Huang, M. Y., Huber, M., Staiger, M., Zhang, L., Hoffmann, A., Lippuner, C. et al.**, Flow cytometry-based high-throughput RNAi screening for miRNAs regulating MHC class II HLA-DR surface expression. *Eur. J. Immunol.* 2022. **52**: 1452–1463.

5 **Shirakawa, F., Yamashita, U. and Suzuki, H.**, Decrease in HLA-DR-positive monocytes in patients with systemic lupus erythematosus (SLE). *J. Immunol.* 1985. **134**: 3560–3562.

6 **Steinbach, F., Henke, F., Krause, B., Thiele, B., Burmester, G. R. and Hiepe, F.**, Monocytes from systemic lupus erythematous patients are severely altered in phenotype and lineage flexibility. *Ann. Rheum. Dis.* 2000. **59**: 283–288.

7 **Spinetti, T., Hirzel, C., Fux, M., Walti, L. N., Schober, P., Stueber, F., Luedi, M. M. et al.**, Reduced monocytic human leukocyte antigen-DR expression indicates immunosuppression in critically ill COVID-19 patients. *Anesth. Analg.* 2020. **131**: 993–999.

8 **Monneret, G., Lepape, A., Voirin, N., Bohe, J., Venet, F., Debard, A. L., Thizy, H. et al.**, Persisting low monocyte human leukocyte antigen-DR expression predicts mortality in septic shock. *Intensive Care Med.* 2006. **32**: 1175–1183.

9 **Handy, J. M., Scott, A. J., Cross, A. M., Sinha, P., O'Dea, K. P. and Takata, M.**, HLA-DR expression and differential trafficking of monocyte subsets following low to intermediate risk surgery. *Anaesthesia* 2010. **65**: 27–35.

10 **Pfortmueller, C. A., Meisel, C., Fux, M. and Schefold, J. C.**, Assessment of immune organ dysfunction in critical illness: utility of innate immune response markers. *Intensive Care Med. Exp.* 2017. **5**: 49.

11 **Diener, C., Keller, A. and Meese, E.**, Emerging concepts of miRNA therapeutics: from cells to clinic. *Trends Genet.* 2022. **38**: 613–616.

12 **Zuckerman, J. E. and Davis, M. E.**, Clinical experiences with systemically administered siRNA-based therapeutics in cancer. *Nat. Rev. Drug Discovery* 2015. **14**: 843–856.

13 **Essandoh, K. and Fan, G. C.**, Role of extracellular and intracellular microRNAs in sepsis. *Biochim Biophys Acta Mol. Basis Dis.* 2014. **1842**: 2155–2162.

14 **Szilagyi, B., Fejes, Z., Pocsi, M., Kappelmayer, J. and Nagy, B., Jr.**, Role of sepsis modulated circulating microRNAs. *EJIFCC* 2019. **30**: 128–145.

15 **Tacke, F., Roderburg, C., Benz, F., Cardenas, D. V., Luedde, M., Hippe, H. J., Frey, N. et al.**, Levels of circulating miR-133a are elevated in sepsis and predict mortality in critically ill patients. *Critical Care Med.* 2014. **42**: 1096–1104.

16 **Wang, H., Zhang, P., Chen, W., Feng, D., Jia, Y. and Xie, L.**, Serum microRNA signatures identified by Solexa sequencing predict sepsis patients' mortality: a prospective observational study. *PLoS One* 2012. **7**: e38885.

17 **Stickel, N., Hanke, K., Marschner, D., Prinz, G., Kohler, M., Melchinger, W., Pfeifer, D. et al.**, MicroRNA-146a reduces MHC-II expression via targeting JAK/STAT signaling in dendritic cells after stem cell transplantation. *Leukemia* 2017. **31**: 2732–2741.

18 **Cazalis, M. A., Friggeri, A., Cave, L., Demaret, J., Barbalat, V., Cerrato, E., Lepape, A. et al.**, Decreased HLA-DR antigen-associated invariant chain (CD74) mRNA expression predicts mortality after septic shock. *Crit. Care* 2013. **17**: R287.

19 **Codolo, G., Toffoletto, M., Chemello, F., Coletta, S., Teixidor, G. S., Battaggia, G., Munari, G. et al.**, Helicobacter pylori dampens HLA-II expression on macrophages via the up-regulation of miRNAs targeting CIITA. *Front. Immunol.* 2020. **10**: 2923.

20 **Agrawal, S. and Kandimalla, E. R.**, Antisense and siRNA as agonists of Toll-like receptors. *Nat. Biotechnol.* 2004. **22**: 1533–1537.

21 **Reynolds, A., Anderson, E. M., Vermeulen, A., Fedorov, Y., Robinson, K., Leake, D., Karpilow, J. et al.**, Induction of the interferon response by siRNA is cell type- and duplex length-dependent. *RNA* 2006. **12**: 988–993.

22 **Cajander, S. C., Tina, E. T., Bäckman, A. B., Magnuson, A. M., Strålin, K. S., Söderquist, B. S. and Källman, J. K.**, Expression of mRNA levels of HLA-DRA in relation to monocyte HLA-DR: a longitudinal sepsis study. *Crit. Care* 2015. **19**: P45.

23 **Lekkou, A., Karakantza, M., Mouzaki, A., Kalfarentzos, F. and Gogos, C. A.**, Cytokine production and monocyte HLA-DR expression as predictors of outcome for patients with community-acquired severe infections. *Clin. Diagn. Lab. Immunol.* 2004. **11**: 161–167.

24 **Ng, P. C., Li, K., Wong, R. P. O., Chui, K., Wong, E., Li, G. and Fok, T. F.**, Proinflammatory and anti-inflammatory cytokine responses in preterm infants with systemic infections. *Arch. Dis. Child Fetal Neonatal Ed.* 2003. **88**: 209–213.

25 **Kim, O. Y., Monsel, A., Bertrand, M., Coriat, P., Cavaillon, J. M. and Adib-Conquy, M.**, Differential down-regulation of HLA-DR on monocyte subpopulations during systemic inflammation. *Crit. Care* 2010. **14**: R61.

26 **Le Tulzo, Y., Pangault, C., Amiot, L., Guilloux, V., Tribut, O., Arvieux, C., Camus, C. et al.**, Monocyte human leukocyte antigen-DR transcriptional downregulation by cortisol during septic shock. *Am J. Respir. Crit. Care Med.* 2004. **169**: 1144–1151.

27 **Portillo, G., Turner, M., Chantry, D. and Feldmann, M.**, Effect of cytokines on Hla-Dr and Il-1 production by a monocytic tumor, Thp-1. *Immunology* 1989. **66**: 170–175.

28 **Giles, A. J., Hutchinson, M. K. N. D., Sonnemann, H. M., Jung, J., Fecci, P. E., Ratnam, N. M., Zhang, W. et al.**, Dexamethasone-induced immunosuppression: mechanisms and implications for immunotherapy. *J. Immunother. Cancer* 2018. **6**.

29 **Friedman, R. C., Farh, K. K. H., Burge, C. B. and Bartel, D. P.**, Most mammalian mRNAs are conserved targets of microRNAs. *Genome Res.* 2009. **19**: 92–105.

30 **Agarwal, V., Bell, G. W., Nam, J. W. and Bartel, D. P.**, Predicting effective microRNA target sites in mammalian mRNAs. *Elife* 2015. **4**: e05005.

31 **Chen, Y. H. and Wang, X. W.**, miRDB: an online database for prediction of functional microRNA targets. *Nucleic Acids Res.* 2020. **48**: D127–D131.

32 **Alvarogracia, J. M., Zvaifler, N. J. and Firestein, G. S.**, Cytokines in chronic inflammatory arthritis .5. mutual antagonism between interferon-gamma and tumor-necrosis-factor-alpha on Hla-Dr expression, proliferation, collagenase production, and granulocyte macrophage colony-stimulating factor production by rheumatoid-arthritis synoviocytes. *J. Clin. Invest.* 1990. **86**: 1790–1798.

33 **Monneret, G., Finck, M. E., Venet, F., Debard, A. L., Bohe, J., Bienvenu, J. and Lepape, A.**, The anti-inflammatory response dominates after septic shock: association of low monocyte HLA-DR expression and high interleukin-10 concentration. *Immunol. Lett.* 2004. **95**: 193–198.

34 **Hekimian, G., Baugnon, T., Thuong, M., Monchi, M., Dabbane, H., Jaby, D., Rhaoui, A. et al.**, Cortisol levels and adrenal reserve after successful cardiac arrest resuscitation. *Shock* 2004. **22**: 116–119.

35 **Woiciechowsky, C., Schoning, B., Lanksch, W. R., Volk, H. D. and Docke, W. D.**, Catecholamine-induced interleukin-10 release: a key mechanism in systemic immunodepression after brain injury. *Crit. Care* 1999. **3**: R107–R111.

36 **Stolk, R. F., Kox, M. and Pickkers, P.**, Noradrenaline drives immunosuppression in sepsis: clinical consequences. *Intensive Care Med.* 2020. **46**: 1246–1248.

37 **Gentile, L. F., Cuenca, A. G., Efron, P. A., Ang, D., Bihorac, A., McKinley, B. A., Moldawer, L. L. et al.**, Persistent inflammation and immunosuppres-

sion: a common syndrome and new horizon for surgical intensive care. *J. Trauma Acute Care Surg.* 2012. **72**: 1491–1501.

38 **Menger, M. D. and Vollmar, B.**, Surgical trauma: hyperinflammation versus immunosuppression? *Langenbecks Arch. Surg.* 2004. **389**: 475–484.

39 **Walseng, E.**, **Bakke, O. and Roche, P. A.**, Major histocompatibility complex class II-peptide complexes internalize using a clathrin- and dynamin-independent endocytosis pathway. *J. Biol. Chem.* 2008. **283**: 14717–14727.

40 **Capasso, M.**, Regulation of immune responses by proton channels. *Immunology* 2014. **143**: 131–137.

41 **Szteyn, K.**, **Yang, W. T.**, **Schmid, E.**, **Lang, F. and Shumilina, E.**, Lipopolysaccharide-sensitive H+ current in dendritic cells. *Am. J. Physiol.: Cell Physiol.* 2012. **303**: C204–C212.

42 **Reth, M.**, Hydrogen peroxide as second messenger in lymphocyte activation. *Nat. Immunol.* 2002. **3**: 1129–1134.

Abbreviations: **APCs**: antigen-presenting cells · **FCM**: flow cytometry · **HLA-DR**: human leukocyte antigen -DR isotype · **miRNA**: microRNA · **qPCR**: quantitative real-time PCR · **SIRS**: systemic inflammatory response syndrome

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