Objective

Monocytes and macrophages express all components of insulin signaling, indicating a functional insulin signaling cascade and development of insulin resistance, usually in the context of systemic insulin resistance. Monocyte/macrophage is a central inflammatory response mediator and is causally associated with pathologic conditions, including type 2 diabetes (T2DM), cardiovascular diseases (CVDs), dyslipidemia, and nonalcoholic fatty liver disease. Pathophysiological significances of monocyte/macrophage insulin signaling have been examined using animal models: in mice, genetic deletion of insulin receptor in myeloid cells results in an anti-inflammatory behavior of macrophages and prevents diet-induced obesity. Consistently, myeloid-specific forkhead transcription factors, inactivated by insulin signaling through phosphorylation, promote atherosclerosis by expanding the granulocyte/macrophage compartment in the bone marrow and peripheral blood. By contrast, with a lipoprotein receptor-deficient background, others reported that myeloid cell insulin action attenuates atherosclerosis by inhibiting the endoplasmic reticulum stress in macrophages. Although the significance is not always consistent among reports, insulin sensitivity in monocytes and/or macrophages plays causal roles in the development of metabolic dysregulation and CVDs. However, it is still unclear whether monocyte/macrophage insulin signaling in humans is affected by T2DM, systemic insulin sensitivity, and other unknown factors.
The present study was designed to clarify whether insulin signaling and pro-inflammatory response of human primary cultured monocytes were altered in individuals with T2DM and without cardiovascular risk factors and to explain factors that affect insulin signaling and pro-inflammatory response in monocytes.

**Research design and methods**

An expanded Methods section is available in the Supplementary Information.

**Study participants**

A total of 53 adult volunteers (Control group) not taking any medication and without history of diabetes, hypertension, dyslipidemia, or atherosclerotic diseases (stroke, ischemic heart, and peripheral arterial diseases) who underwent a medical check-up at Isawa Hot Spring Hospital and 59 in patients with T2DM (T2DM group) at the University Hospital of Yamanashi were included. The study was approved by the certified review board of the University of Yamanashi (#1668) and Isawa Hot Spring Hospital (#2018-003). Written informed consent was obtained from all participants.

**Monocyte isolation**

About 25 mL of the fasting anti-coagulated blood sample was diluted with an equal volume of lymphocyte separation solution (Nakalai tesque, Japan). The samples were centrifuged at 400 g, in a swing out rotor for 30 min at 22°C, and the peripheral blood mononuclear cell (PBMC) layers were harvested using a pipette. PBMCs were repeatedly washed using Dulbecco’s Modified Eagle Medium (DMEM) (Thermo Fisher Scientific Inc., Waltham, MA, USA) with 1% penicillin/streptomycin (P/S) containing 10% fetal bovine serum (FBS). PBMCs were cultured on collagen-coated multi-well plates in DMEM with 1% P/S containing 10% FBS for 2 h in an incubator. PBMC was washed with phosphate-buffered saline (PBS) to remove lymphocytes.

Purity of the monocytes was verified by a FACSCelesta (Becton, Dickinson and Company, NJ, USA), using phycoerythrin (PE)-conjugated anti-human CD14 Antibody (BioLegend, CA, USA) (Supplemental Figure 1). For serum starvation, the media was replaced with DMEM containing 0.25% bovine serum albumin (BSA) for 2 h. Cultured monocytes were stimulated by 50 nM insulin for 5 min or 10 ng/mL LPS for 4 h on 12-well plates.

**Quantitative real-time polymerase chain reaction (qRT-PCR) analysis**

Total RNA of cultured human monocytes was isolated using TRizol (Thermo Fisher Scientific Inc., Waltham, MA, USA) as previously described. RNA was reverse transcribed with Random Primer (Thermo Fisher Scientific Inc., Waltham, MA, USA) and ReverTra Ace (Toyobo Co., Ltd., Osaka, Japan). Quantitative real-time polymerase chain reaction (RT-PCR) was conducted using ViiaTM 7 Real-Time PCR Systems with Fast SYBR Green Master Mix Reagent (Thermo Fisher Scientific Inc.). Primer sequences are available on request. Data were normalized to the ACTB levels and analyzed using the comparative CT and relative quantification method. LPS-stimulated elevation of gene expression assessed as fold induction was used for analyses. Sample sizes of gene expression analysis varied in target genes because unsuccessful PCR samples were excluded from analysis. The sample sizes for gene expression analysis varied in one target gene to the other. Sample sizes (n) were mentioned in each Figure.

**Western blotting**

Samples (15 µg each) were mixed with the loading buffer, heated at 95°C for 5 min and resolved using 8% Tris-Glycine gels (Thermo Fisher Scientific Inc., Waltham, MA, USA) electrophoresis (SDS-PAGE). Cellular proteins were transferred to the Immuno-Blot polyvinylidene fluoride (PVDF) membrane (Bio-Rad Laboratories, USA) by electroblotting. The membranes were blocked with a PVDF blocking reagent (Toyobo Life Science, Japan) for 1 h, followed by an overnight incubation with 1:1000 diluted primary antibodies (pAKT, AKT, pFOXO3a, and FOXO3a). All primary antibodies were purchased from Cell Signaling (Cell Signaling Technology, Inc., USA). Blots were washed three times with PBS with Tween® 20 (PBS-T) and incubated for 1 h with horseradish peroxidase-conjugated secondary antibody (Promega, Madison, WI, USA). Band intensities were measured using an image analysis software (NIH Image J software). Monocyte insulin sensitivity was represented as “insulin-stimulated pAKT” and “insulin-stimulated pFOXO3.” The “insulin-stimulated pAKT” was calculated as follows: “insulin-stimulated pAKT”=(the ratio of pAKT to total AKT after insulin stimulation)/(the ratio of pAKT to total AKT before insulin stimulation). Data pertaining to insulin-stimulated pAKT and pFOXO3 were successfully obtained from 53 to 47 subjects belonging to the control group, and from 59 to 31 subjects belonging to the T2DM group. Sample sizes (n) were mentioned in each Figure.

**Statistical analysis**

Continuous variables were reported as mean values ± one standard error or as median and interquartile range if not normally distributed, whereas categorical variables were reported as numbers and percentages. Differences between the two groups were tested using the Student’s t-test for normally distributed unpaired data once (Kolmogorov–Smirnov test); otherwise, a nonparametric
test (Mann–Whitney U-test) was used. Wilcoxon matched-pair signed-rank tests (two-tailed) were used to analyze each pairwise comparison within each group. Categorical variables were analyzed using the χ² test or Fisher’s exact test as necessary. Correlations between quantitative variables were investigated using Spearman’s test. Data are expressed as mean with standard deviations or box-and-whisker plots. Multiple linear regression analysis was employed to determine factors that were independently associated with a dependent variable. Statistical analysis was conducted using the GraphPad Prism 8 (GraphPad software, Inc., CA, USA). A p-value of <0.05 was used to assess the significance of all statistical analyses.

Results

Participant characteristics

Table 1 lists the clinical characteristics of the control and T2DM groups. No significant differences were observed in gender, age, smoking, diastolic blood pressure, and estimated glomerular filtration rate between the two groups. Body mass index, systolic blood pressure, blood glucose level, and serum IRI level, HOMA-IR, and lipid profiles were significantly different between the two groups. Eleven patients in T2DM group had history of atherosclerotic diseases, median ABI, and maximum carotid IMT, which were available only in the T2DM group, were within normal ranges. A total of 34, 19, and 33 patients had diabetic
neuropathy, retinopathy, and nephropathy, respectively. Fifty patients, including 23 insulin users, in the T2DM group were taking diabetes medications.

**Impaired insulin signaling in cultured monocytes from T2DM patients**

Gene expression analyses of primary cultured monocytes revealed significant downregulation of INSR and IRS1 expression in T2DM compared with that in the control group (Figure 1). IRS2, FOXO1, and FOXO3 expressions were comparable between the two groups. Protein analyses of primary cultured monocytes showed that insulin-stimulated pAKT and pFOXO3 was significantly suppressed in T2DM compared with that in the control group (Figure 2(a)), indicating insulin resistance of monocytes in T2DM. Moreover, monocytes from patients with T2DM with diabetic nephropathy or history of atherosclerotic diseases suppressed insulin-stimulated pAKT (Figure 2(b)). Insulin-stimulated pAKT was significantly correlated that of pFOXO3 in the control and T2DM groups (Supplemental Figure 2). These data suggest that T2DM impairs insulin signaling of monocytes, associated with downregulation of INSR and IRS1 gene expression.

Gender and smoking did not affect monocyte insulin sensitivity in the control and T2DM groups (Supplemental Figure 3). The presence of hypertension, which was included only in the T2DM group, also did not alter monocyte insulin sensitivity.

### Table 1. Participant characteristics.

|                          | Control     | T2DM        | p value |
|--------------------------|-------------|-------------|---------|
| Subjects (n)             | 53          | 59          |         |
| Female (n, %)            | 24 (45)     | 25 (42)     | 0.85    |
| Age (years)              | 62 ± 8      | 63 ± 12     | 0.76    |
| Duration of diabetes (years) | 0          | 10 (4–18)   |         |
| Smoking (n, %)           | 21 (40)     | 31 (53)     | 0.19    |
| Diabetic complications (n, %) |             |             |         |
| Neuropathy               | 0 (0)       | 34 (58)     |         |
| Retinopathy              | 0 (0)       | 19 (32)     |         |
| Nephropathy              | 0 (0)       | 33 (56)     |         |
| Atherosclerotic diseases | 0 (0)       | 11 (19)     |         |
| BMI (kg/m²)              | 22.5 ± 2.8  | 26.3 ± 5.5  | <0.001  |
| SBP (mmHg)               | 118 ± 10    | 132 ± 19    | <0.001  |
| DBP (mmHg)               | 74 ± 9      | 74 ± 13     | 0.87    |
| FPG (mg/dL)              | 96 ± 8      | 165 ± 56    | <0.001  |
| HbA1c (%)                | 5.6 ± 0.3   | 9.6 ± 2.2   | <0.001  |
| IR (µU/mL)               | 2.8 ± 1.5   | 6.4 ± 3.7   | <0.001  |
| HOMA-IR                  | 0.67 ± 0.38 | 2.41 ± 1.41 | <0.001  |
| TG (mg/dL)               | 93 ± 53     | 157 ± 89    | <0.001  |
| HDL-C (mg/dL)            | 62 ± 15     | 49 ± 16     | <0.001  |
| LDL-C (mg/dL)            | 120 ± 28    | 130 ± 46    | 0.25    |
| AST (U/L)                | 21 ± 5      | 27 ± 18     | <0.05   |
| ALT (U/L)                | 20 ± 9      | 30 ± 23     | <0.01   |
| γ-GT (U/L)               | 25 ± 14     | 61 ± 129    | <0.05   |
| eGFR (mL/min/1.73 m²)    | 72 ± 11     | 74 ± 19     | 0.53    |
| ABI N/A                  | 1.09 (1.03–1.14) |         |         |
| baPWV (cm/s)             | 1673 (1430–3261) |         |         |
| Maximal IMT (mm)         | 1.15 (0.90–1.53) |         |         |
| Medication (n, %)        |             |             |         |
| Antidiabetic             | 0 (0)       | 50 (84)     |         |
| Antilipidemic            | 0 (0)       | 24 (41)     |         |
| Antihypertensive         | 0 (0)       | 27 (46)     |         |

BMI: body mass index; SBP: systolic blood pressure; DBP: diastolic blood pressure; FPG: fasting blood glucose; HbA1c: hemoglobin A1c; IRI: immuno-reactive insulin; HOMA-IR: homeostasis model assessment of insulin resistance; TG: triglycerides; HDL-C: high-density lipoprotein cholesterol; LDL-C: low-density lipoprotein cholesterol; AST: aspartate transaminase; ALT: alanine transaminase; γ-GT: γ-glutamyl transferase; eGFR: estimated glomerular filtration rate; ABI: ankle-brachial index; baPWV: brachial-ankle pulse wave velocity; IMT: intima-media thickness; N/A: not available.

Data were expressed as mean ± SEM, or median and interquartile range. Data were compared using student’s t test, or the Mann-Whitney U test.
Insulin resistance increased with age in cultured monocytes of healthy participants

In the control group, insulin-stimulated pAKT was significantly correlated with BMI, HbA1c, IRI, and HOMA-IR (Figure 3). Similarly, insulin-stimulated pFOXO3 was significantly correlated with BMI, HbA1c, and FPG and showed a trend of correlation for HOMA-IR (Supplemental Figure 4). By contrast, insulin-stimulated pAKT was not significantly correlated with BMI, HbA1c, FPG, IRI, or HOMA-IR in the T2DM group (data not shown). Significant negative correlation between age and insulin-stimulated pAKT and pFOXO3 was commonly observed in both control and T2DM groups (Figure 4(a) and (b)). Furthermore, regression line slopes between age and insulin-stimulated pAKT and pFOXO3 were milder in T2DM than those in the control group (Figure 4(a) and (b)). These data suggest that insulin signaling in monocytes reflects systemic glucose metabolism and insulin sensitivity in the control group, and that aging develops insulin resistance in monocytes, possibly irrespective of medication, diabetic conditions, or other comorbidities in the T2DM group.

We further performed a multiple regression analysis to clarify whether age independently affects monocyte insulin sensitivity, using insulin-stimulated pAKT as a dependent variable. Independent variables were gender and age, and factors related to systemic insulin resistance such as BMI, and HOMA-IR. Age, BMI, and HOMA-IR were significant predictors of monocyte insulin sensitivity in control subjects, and only age was a significant predictor in T2DM subjects (Table 2). In all subjects, age and BMI were selected as significant predictors. It suggests that age independently reduces monocyte insulin sensitivity irrespective to presence of T2DM.

Association of insulin resistance with inflammatory responses in cultured monocytes

Finally, we assessed inflammatory responses in cultured monocytes, recognized as a crucial cellular component to promote atherosclerosis, via LPS-stimulated induction of inflammatory genes. In the control group, LPS-stimulated induction of TNFA, and NOS2 was significantly and negatively correlated with insulin-stimulated pAKT (Figure 5(a)). Age was also significantly correlated with LPS-stimulated induction of TNFα, and showed a trend of correlation to LPS-stimulated IL6 induction (Figure 5(b)). By contrast, insulin-stimulated pAKT did not show significant correlation to LPS-stimulated induction of TNF, NOS2, or IL6 in the T2DM group (Supplemental Figure 5). Age was not correlated with LPS-stimulated TNF, NOS2, or IL6 induction in T2DM (data not shown). These data suggest that age and monocyte insulin resistance enhance pro-inflammatory responses in monocytes, even in subjects without cardiovascular risk factors.

Discussion

Age-associated monocyte insulin resistance irrespective of cardiovascular risk factors

Previous studies indicated that macrophages and monocytes express insulin receptors. In the present study, Spearman’s correlation analysis showed that insulin-stimulated pAKT and
pFOXO3 were negatively associated with age. Furthermore, multiple regression analysis revealed that age independently reduced monocyte insulin sensitivity irrespective of presence of T2DM. It suggests that aging itself is a major factor to determine insulin sensitivity in human monocytes, independent of the pharmacological intervention and comorbidities in the T2DM group. Whereas systemic insulin resistance assessed as serum IRI levels and HOMA-IR is markedly higher in the T2DM group than that in the control group, monocyte insulin sensitivity does not always reflect the difference: the difference of insulin-stimulated induction in AKT phosphorylation between the two groups becomes smaller with aging, suggesting that aging may diminish the difference of insulin sensitivity in monocytes between the two groups, consequently equalizing the risk of diseases related to insulin signaling of monocyte/macrophage between the elderly healthy and diabetic participants. Indeed, some major algorithms designed to provide relative individual risk scores [Framingham,13 QRISK®2-2016,14 ACC/AHA ASCVD15] have concluded that the strongest risk factor for atherosclerosis and CVD is indisputably age; using these existing algorithms, simply increasing age—while holding all other variables constant—results in a marked increase in the risk for CVD.16–19

**Age- and systemic insulin sensitivity-associated change of monocyte insulin sensitivity among healthy individuals**

The present study suggests that even in the monocytes from the control participants who are without any cardiovascular risk factors or medications, inflammatory responses were negatively associated with insulin sensitivity. Given that insulin sensitivity in monocytes was correlated with fasting IRI level and HOMA-IR in the control group, the risk of diseases related to insulin signaling and inflammatory responses of monocyte/macrophage may continuously increase with systemic insulin resistance, even in participants without apparent risk factors. Investigators in the Insulin Resistance Atherosclerosis Study showed that insulin resistance was directly related with atherosclerosis,20 and a follow-up prospective study reported insulin resistance as an important risk factor for CVD.21 A meta-analysis of 65 studies also revealed that insulin resistance, assessed by HOMA-IR, was a good predictor for CVD.22 Using the Archimedes model and a population representative of young nondiabetic adults aged 20–30, the authors concluded that preventing insulin resistance could avoid
approximately 42% of myocardial infarctions during a simulated follow-up period of 60 years.\textsuperscript{23} Taken together, the difference of monocyte insulin resistance in the control group may reflect that of risk for CVDs. However, it is still unclear whether enhancing monocyte insulin sensitivity with interventions reduces the risk of atherosclerosis in participants without risk factors. Further studies are needed to clarify the pathophysiological significance of the “subclinical” insulin resistance in monocytes.

In the present study, we were unable to compare the absolute levels of phosphorylation in AKT and FOXO3 before and after insulin stimulation because we could not perform Western blotting simultaneously for all the samples to perform relative comparisons between subjects. Moreover, we could not include control samples on each blot to enable absolute quantitation of the extent of phosphorylation. Then, the significance of absolute activities of AKT and FOXO3, especially in distinction to basal and insulin-stimulated activities of insulin signaling, remains unclear. It has been reported that macrophages from transgenic mice expressing constitutively

![Figure 4](image-url)

**Figure 4.** Correlation of insulin-stimulated AKT or FOXO3 phosphorylation with age in cultured human monocytes: univariate correlations between insulin-stimulated pAKT and age in cultured monocytes of the (a) control (white circles) and (b) T2DM (black circles) groups. Spearman correlation coefficients ($r$), their corresponding $p$-values, and regression slopes are shown.

**Table 2.** Multiple regression analysis.

| Variable | Estimate (SE) | t | VIF | $p$ |
|----------|--------------|---|-----|----|
| **Control subjects ($R^2 = 0.34, F = 6.26, p < 0.001$)** | | | | |
| Gender | 0.32 (0.62) | 0.52 | 1.01 | 0.61 |
| Age | −0.11 (0.12) | 2.84 | 1.01 | <0.01 |
| BMI | −0.24 (0.12) | 2.02 | 1.15 | <0.05 |
| HOMA-IR | −2.21 (0.86) | 2.58 | 1.14 | <0.05 |
| **T2DM subjects ($R^2 = 0.36, F = 4.34, p < 0.01$)** | | | | |
| Gender | 0.31 (0.33) | 0.93 | 1.21 | 0.36 |
| Age | −0.05 (0.01) | 3.62 | 1.20 | <0.01 |
| BMI | −0.02 (0.04) | 0.58 | 1.33 | 0.57 |
| HOMA-IR | −0.05 (0.12) | 0.41 | 1.31 | 0.68 |
| **All subjects ($R^2 = 0.21, F = 6.27, p < 0.001$)** | | | | |
| Gender | 0.26 (0.40) | 0.65 | 1.02 | 0.52 |
| Age | −0.08 (0.02) | 4.21 | 1.04 | <0.001 |
| BMI | −0.13 (0.05) | 2.53 | 1.33 | <0.05 |
| HOMA-IR | −0.08 (0.10) | 0.80 | 1.30 | 0.43 |

SE: standard error; $F$: variance ratio; VIF: variance inflation factor. Gender is coded as 1 = male and 0 = female.
active Akt display higher levels of lipopolysaccharide-induced IL-10 expression.\textsuperscript{24} Additionally, genetic ablation of the 3 genes encoding FoxO isoforms 1, 3a, and 4, in myeloid cells, which mimics constitutive inactivation of FoxOs by Akt, reportedly increases inducible nitric oxide synthase (iNOS) expression and oxidative stress to promote atherosclerotic lesion formation in low-density lipoprotein receptor knockout mice.\textsuperscript{2} It therefore suggests that, basal activity level of insulin signaling in monocytes/macrophages may be involved in regulation of inflammatory signals. The pathophysiological significance of basal level and insulin-stimulated activity of insulin signaling in monocytes/macrophages remains to be clarified.

Possible mechanisms of age-associated decline of monocyte insulin sensitivity

The molecular mechanisms that aging suppresses insulin sensitivity in monocytes remain unveiled in the present study. In telomerase reverse transcriptase-deficient mice, a model of premature aging caused the accelerated telomere shortening due to telomerase deficiency,\textsuperscript{25,26} high-fat and high-sucrose diet promoting aging of the adipose tissue represented by p53 accumulation, which was associated with the permeation of the inflammation cell, increased expression of pro-inflammatory genes and insulin resistance.\textsuperscript{27} These observations are consistent with our data that LPS-stimulated induction of pro-inflammatory gene expression was negatively correlated with insulin sensitivity in monocytes of the control group. In our data, no differences in the gene expression of \textit{TP53}, which encodes P53 in humans, were observed between the two groups. As well, there was no correlation between the gene expression of \textit{TP53} and insulin sensitivity, BMI, HbA1c, IRI, or HOMA-IR (data not shown). Nevertheless, regulation of p53 activity is generally thought to mainly involve post-translational modifications, including ubiquitination, phosphorylation, acetylation, and methylation.\textsuperscript{28–30} Then, P53 may contribute to age-dependent decreased insulin sensitivity and enhanced pro-inflammatory responses in monocytes as observed in the present study. Furthermore, as described above, the association between age and absolute levels of phosphorylation in AKT and FOXO3 before and after insulin stimulation cannot be discussed in the present study. However, in 3T3-L1 adipocytes, it has been reported that pharmacological activation of p53 by nutlin-3 inhibits insulin-stimulated phosphorylation of insulin receptor and Akt, without affecting baseline phosphorylation levels of them.\textsuperscript{31} Then, it is possible that, if P53

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\textbf{Figure 5.} Correlation of insulin-stimulated AKT phosphorylation or age with inflammatory gene expression in cultured human monocytes of the control group: univariate correlations between the (a) insulin-stimulated pAKT or (b) age and TNF, NOS2, and IL6 induction in cultured monocytes of the control group. Spearman correlation coefficients ($r$) and their corresponding $p$-values are shown.
mediates the age-associated decline of monocyte insulin sensitivity as observed in the present study, it may mainly affect activity of AKT after insulin stimulation. Cellular and molecular mechanisms of age-associated development of insulin resistance and pro-inflammatory characteristics in monocytes require further studies.

In the present study, gene expression levels varied among individuals: individual variations of INSR mRNA levels showed approximately 30-fold range. In a paper analyzing INSR mRNA levels in human liver biopsies from 11 healthy individuals using qRT-PCR with comparative CT and relative quantification method, 32 individual variation of the gene expression levels looks approximately 20-fold range by estimation from box and whisker plots in the paper. Additionally, by estimation from dot plots in a paper showing INSR mRNA levels in 16 human normal breast tissues using qRT-PCR, individual variation of the gene expression levels looks approximately 50-fold range. 33 Although there is no exactly comparable study using human monocytes, it is conceivable that basal gene expression levels of INSR in human tissues vary among individuals as observed in monocytes of the present study.

In conclusion, our observation suggests that aging independently plays an important role in the development of monocyte insulin resistance, not only in patients with DM but also in healthy participants. Monocyte insulin sensitivity is negatively correlated with inflammatory responses and may be helpful for subclinical risk assessment of CVDs and/or insulin resistance in participants without risk factors.

List of abbreviations

BMI, body mass index; SBP, systolic blood pressure; DBP, diastolic blood pressure; FPG, fasting blood glucose; HbA1c, hemoglobin A1c; IR1, Immuno-reactive Insulin; HOMA-IR, homeostasis model assessment of insulin resistance; TG, triglycerides; HDL-C, high-density lipoprotein cholesterol; LDL-C, low-density lipoprotein cholesterol; AST, aspartate transaminase; ALT, alanine transaminase; γ-GT, γ-glutamyl transferase; eGFR, estimated glomerular filtration rate; ABI, ankle-brachial index; IMT, intima-media thickness.

Declaration of conflicting interests

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Ethics approval and consent to participate

The study was approved by the certified review board of the University of Yamanashi (#1668) and Isawa Hot Spring Hospital (#2018-003). Written informed consent was obtained from all participants.

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Supplemental material

Supplemental material for this article is available online.

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