

Predictive hazard markers for the ranking of pulmonary toxicity of nanomaterials

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Abstract

Background

We analyzed the mRNA expression of chemokines in rat lungs following intratracheal instillation of nanomaterials in order to find useful predictive markers of the pulmonary toxicity of nanomaterials. Four nanomaterials, nickel oxide (NiO) and cerium dioxide (CeO₂) as nanomaterials with high pulmonary toxicity, and titanium dioxide (TiO₂) and zinc oxide (ZnO) as nanomaterials with low pulmonary toxicity, were intratracheally administered into rat lungs at 0.8 mg/kg BW or 4 mg/kg BW as low and high doses, respectively. Five chemokine genes (*C-X-C motif chemokine 5 (CXCL5)*, *C-C motif chemokine 2 (CCL2)*, *C-C motif chemokine 7 (CCL7)*, *C-X-C motif chemokine 10 (CXCL10)*, and *C-X-C motif chemokine 11 (CXCL11)*) were selected using cDNA microarray analysis at 1 month after instillation of NiO in the high dose group. The mRNA expression of these 5 genes in the lung tissues were evaluated at 3 days, 1 week, 1 month, 3 months and 6 months after instillation of the 4 nanomaterials, using real-time quantitative polymerase chain reaction (RT-qPCR) during the observation periods.

Results

An increased expression of *CXCL5* in the NiO and CeO₂ groups was persistently higher than in the control group, and that in the TiO₂ and ZnO groups was transiently higher than in the control group. *CCL2* and *CCL7* also showed an increasing tendency similar to *CXCL5*, although there was not a persistent significance. The receiver operating characteristic (ROC) results of nanomaterial toxicity with the expression level of each gene showed a considerable relationship between the pulmonary toxicity ranking of nanomaterials and the expression of *CXCL5*, *CCL2* and *CCL7*, with area under the curves (AUC) of 0.95 or higher at 1 week and 1 month. The expression levels of these 3 genes also moderately or strongly correlated with the degree of inflammatory cells in the lung tissues.

Conclusion

Our results suggest that 3 chemokine genes, especially *CXCL5*, followed by *CCL2* and *CCL7*, can be useful as biomarkers for the ranking of the pulmonary toxicity of nanomaterials.

Background

Nanoparticles are defined as particles with at least one dimension of 100 nm or less [1, 2], and in recent years the demand for industrial nanomaterials composed of these nanoparticles has increased significantly. New industrial nanomaterials are being created one after another by nanotechnology for controlling functions on the nanometer scale, and are being used for various applications in various fields. The pulmonary toxicity of nanomaterials needs to be fully assessed before the industrial nanomaterials are handled by humans. It is necessary to develop biomarkers to predict the hazard level

of industrial nanomaterials, because multi-walled carbon nanotubes, a representative nanomaterial, are known to be carcinogenic: the development of lung tumors or malignant pleural mesothelioma was confirmed in intratracheal instillation [3, 4] and in inhalation studies [5].

In pulmonary disorders caused by respirable chemicals, it is considered that the chemical deposits in the lungs cause sustained inflammation and ultimately the formation of chronic and irreversible lesions such as lung fibrosis and tumors [6–10]. It has been reported, for example, that asbestos and crystalline silica, which have high pulmonary toxicity, cause sustained inflammation in the lungs, leading to irreversible fibrosis, lung cancer and mesothelioma [11, 12]. Chemicals with low pulmonary toxicity, on the other hand, such as micron-sized TiO₂, do not cause inflammation, or cause only transient inflammation, and then do not progress to fibrosis or tumor formation in the lung [13–15]. Thus, sustained inflammation is considered to be an important process in the induction of chronic and irreversible lesions of the lung [6–10, 16]. Multiwalled carbon nanotubes, which are carcinogenic, have shown sustained inflammation in the lung following inhalation and intratracheal instillation [17–20], while TiO₂ nanoparticles that did not cause tumors did not induce inflammation in an inhalation exposure study, and caused only transient inflammation in an intratracheal instillation study [21]. Taken together, it is thought that ‘sustained inflammation’ is an important process in predicting lung disorders caused by industrial nanomaterials. It is considered that the detection of biomarkers that reflect sustained inflammation in the lung can lead to early detection of the hazardous effects of nanomaterials, and can thus predict the progression to chronic/irreversible lesions.

In this study, we focused on inflammation-related genes based on the results of a comprehensive gene expression analysis using cDNA microarray, and examined whether or not biomarkers for predicting lung disorder by nanomaterials can be detected following the intratracheal instillation of nanomaterials with different pulmonary toxicities.

Result

Gene expression analysis

Table 1 shows the results of gene expression in the NiO-high dose group by cDNA microarray at 1 month after intratracheal instillation. The number of genes whose expression was increased eight times or more was 16 (Table 1(A)), and 5 genes among them were chemokine genes ((C-X-C motif chemokine 5 (*CXCL5*), C-C motif chemokine 2 (*CCL2*), C-C motif chemokine 7 (*CCL7*), C-X-C motif chemokine 10 (*CXCL10*), and C-X-C motif chemokine 11 (*CXCL11*)) (Table 1(B)). Figure 1 and Additional file 1 (Table S1) show the validated expression levels of the 5 chemokine genes induced by the 4 nanomaterials following intratracheal instillation using qRT-PCR over the observation time.

The gene expression of *CXCL5* in the lung tissue exposed to NiO and CeO₂, which have high pulmonary toxicity, was persistently high compared with the negative control throughout the observation time, while the expression was increased transiently or not increased in the lung tissue exposed to TiO₂ and ZnO,

which have low pulmonary toxicity, during the observation time. The nanomaterials with high pulmonary toxicity induced persistent expression patterns of the *CXCL5* gene during the observation period, and the nanomaterials with low pulmonary toxicity did not, or had transient patterns during the observation period.

The expression patterns of *CCL2* and *CCL7* also showed a similar tendency to *CXCL5*. *CCL2* genes had a significantly persistent increase in the NiO and CeO₂ high dose groups, and *CCL7* genes had a significantly persistent increase in the NiO high dose group. Both genes had an insignificant increase in the NiO and CeO₂ low dose groups and in the CeO₂ high dose group. There was a transient increase in the TiO₂ and ZnO-exposed groups at 3 days or 1 month following intratracheal instillation.

On the other hand, the gene expression of *CXCL10* and *CXCL11* in the NiO and ZnO-exposed groups increased transiently at 3 days or 1 week or 1 month following intratracheal instillation, and no difference was observed in the expression level between nanomaterials of high and low pulmonary toxicity.

Assessment of the accuracy of gene expression of the toxicity of chemicals

Table 2 shows the results of the receiver operating characteristics (ROC) for the toxicity of the nanomaterials by the gene expression. Recognizing NiO and CeO₂ as substances with high pulmonary toxicity and TiO₂ and ZnO as substances with low pulmonary toxicity, we examined whether or not this pulmonary toxicity ranking was related to the expression of the 5 chemokine genes. The maximum areas under the curves (AUC) of each gene were generally observed at 1 week and 1 month following intratracheal instillation. There was a considerable relationship between the pulmonary toxicity of the nanomaterials and the gene expression of *CXCL5*, *CCL2* and *CCL7*, with AUC of 0.95 or higher at 1 week and 1 month. Further, false negatives could be minimized when evaluated with the combination of *CXCL5*, *CCL2*, and *CCL7* (Table 3). On the other hand, there seemed to be a relatively low relationship between the pulmonary toxicity of the nanomaterials and gene expressions of *CXCL10* and *CXCL11* during the other observation times.

CXCL5, CCL2 and CCL7 immunostaining

Figure 2 shows CXCL5, CCL2 and CCL7 immunostaining in the NiO-high dose group and the negative control group (distilled water) at 1 month after intratracheal instillation. The CXCL5 immunostaining positive cells were observed mainly at the gathering sites of inflammatory cells, mainly macrophages, at 1 month in the high dose of NiO exposure group. Similar to CXCL5, positive cells of CCL2 and CCL7 immunostaining were observed in aggregations of inflammatory cells centered on macrophages.

Pathological features in the rat lungs

Figure 3 shows the inflammatory cell infiltration scores of the pathological features in the rat lungs. There was sustained inflammation in the rat lungs exposed to NiO and CeO₂, while only transient inflammation was observed in rat lungs exposed to TiO₂ and ZnO. In figure 4, pulmonary inflammation appears mainly

in the macrophages and neutrophils in the rat lungs exposed to NiO, and in the macrophages in the rat lungs exposed to CeO₂, respectively. Figure 5 shows the relationship between the inflammatory cell score and the gene expression of *CXCL5*, *CCL2* and *CCL7* in the lung exposed to the nanomaterials. The expression levels of *CXCL5*, *CCL2* and *CCL7* at 1 week and 1 month correlated moderately or strongly with the degree of inflammatory cell infiltration in the lung tissue.

Discussion

According to the microarray analysis in our experiments, the inflammation-related genes that showed upregulation in the lungs of rats injected intratracheally with NiO nanoparticles were *CXCL5*, *CCL2*, *CCL7*, *CXCL10* and *CXCL11*, all of which are chemokine genes. All these chemokines are involved in inflammatory responses through the migration, accumulation and activation of inflammatory cells such as neutrophils and macrophages [22-25].

CXCL5 is a CXC chemokine with a glutamate-leucine-arginine (ELR) motif (ELR + chemokine) and has potent chemotactic and activating functions of neutrophils in the lung [26]. In our experiments, the gene expression of *CXCL5* was persistently increased by NiO and CeO₂, which have high pulmonary toxicity, and not, or only transiently, increased by TiO₂ and ZnO, which have low pulmonary toxicity (Figure 1). In other studies, the expression of *CXCL5* by respirable chemicals was enhanced in the lungs of rodents exposed to single-wall carbon nanotubes and inflammogenetic stainless-steel welding fumes [27-28]. For lung lesion other than pulmonary inflammation, exposure to cigarette smoke, which leads to lung cancer and COPD, also induced *CXCL5* expression in the lung [29]. Alternatively, no up-regulation of *CXCL5* was reported in rat lungs exposed to C60 fullerenes with low toxicity [30].

The chemokines *CCL2* and *CCL7* are known as chemotactic agents for monocytes and have been found to play a key role in mediating lung inflammation. It is also known that *CCL2* and *CCL7* are both CC chemokines, are closely related to each other, have a common receptor (CCR2), and elicit similar responses, such as those involved in the migration of macrophages, lymphocytes, and neutrophils [31]. Similar to *CXCL5* in our experiment, exposure to NiO and CeO₂ showed a continuous, increasing trend of the expression of *CCL2* and *CCL7*, but exposure to TiO₂ and ZnO did so only transiently (Figure 1). Langley et al. found that *CCL2* and *CCL7* were persistently upregulated in rat lungs exposed to inhaled crystalline silica, a dust with high toxicity [32]. Fujita et al. also reported that *CCL2* and *CCL7* were upregulated in the lungs of rats injected intratracheally with inflammogenetic SWCNT [27, 33]. Abdelgied et al. found that *CCL2* expression was increased in rat lungs following intratracheal instillation of potassium octatitanate fibers (POT fibers), which are suggested to have carcinogenic potential [34]. On the other hand, it has also been reported that *CCL2* and *CCL7* expression was transient in rat lungs following intratracheal instillation of C60 fullerenes with low toxicity [30].

The expression of *CXCL5*, *CCL2* and *CCL7* had a certain level of correlation with the lung inflammation score in our experiment, and these findings suggested that these gene expressions were involved in lung inflammation as the main pathological lesion from exposure to nanomaterials. Immunostaining results

revealed that CXCL5, CCL2 and CCL7 stained mainly macrophages in the infiltration of inflammatory cells in the alveolar space, although only lung tissues exposed to NiO were examined. This suggests that these chemokines in the alveolar space also contributed to the formation of pulmonary inflammation.

CXCL10 and CXCL11 are ELR- CXC chemokines that have CXCR3 as a common receptor [35]. No difference in the expression level between nanomaterials of high and low pulmonary toxicity was observed in our experiment. Fundamentally, CXCL10 and CXCL11, which are Th1 network-related cytokines, seem to have little relation to this pathological condition in which the inflammatory cells are mainly macrophages and neutrophils. T cells were rarely involved in the lesions in the present experiment, which is the reason why these genes expressions are not reflected in the ranking.

There was a considerable relationship between the toxicity ranking of the nanomaterials and the expression of *CXCL5*, *CCL2* and *CCL7*, which have AUC with high values. Under the condition of high AUC, low doses of highly toxic substances produced more gene expression than high doses of less toxic substances. The dosage settings in this experiment, 0.8 mg / kg BW and 4 mg / kg BW, were necessary for considering the validity of the expression level of the 3 genes. We considered that the low and high dosages were approximately the minimum and maximum doses necessary to evaluate the pulmonary toxicity of the metal oxide nanoparticles in our experiment. The low dose is approximately the minimum dose at which nanomaterials with high toxicity induced pulmonary inflammation. We previously injected NiO at 0.8 mg/kg BW in rats of a different species than those in the present experiment, and there was similar mild neutrophil inflammation [10]. The dose of 4 mg/kg BW was considered to be the maximum dose that did not cause overload in intratracheal instillation studies of nanoparticles. We previously reported that doses in excess of 4 mg/kg induced pulmonary surplus inflammation and delay of the biological half time of nanoparticles [36]. Morrow PE et al. and Bellmann B et al. reported in toner studies, as well, that a delayed clearance of alveolar macrophages occurred between 1 mg/rat (4 mg/kg BW in our experiment is equivalent to 1mg/rat) and 3 mg/rat of lung deposition [37, 38], indicating that the threshold of overload is between 1 and 3 mg/rat. It was speculated from these data that exposure to doses above 1 mg/rat might induce pulmonary toxicity by the chemicals themselves as well as toxicity from the excessive dose. These doses, 0.8 mg/kg BW and 4 mg/kg BW, as the burden on the lungs of nanomaterials after intratracheal instillation, may correspond to approximately 0.36 of 1.8 years of the inhalation period at a concentration of 3 mg/m³, as the maximum concentration for humans, of inhalable dust without crystalline silica (working time 8 hours/day, 5 days/week).

As for the examination of the observation times, there were high AUCs between the pulmonary toxicity of the nanomaterials and the gene expression of the 3 chemokines at 1 month and 1 week following intratracheal instillation. In the acute phases, for example at and around 3 days after intratracheal instillation, there may have been a bolus effect in which even low-toxic substances induced pulmonary inflammation. Yoshiura et al. reported that even TiO₂ (P90), which did not cause pulmonary tumors in 2 years observation time following intratracheal instillation, induced transient inflammation at 3 days in the same intratracheal instillation study [39]. ZnO is known to be a soluble metal that causes inflammation, and in our experiment, ZnO nanoparticles, among the 4 nanomaterials studied, induced severe pulmonary

inflammation at 3 days following intratracheal instillation. At 1 month, the pulmonary inflammation by the bolus effect had disappeared, and the gene may have been expressed due to the toxicity of the original nanomaterial. Although some inflammation remained in the low toxic substance exposure group at 1 week (Figure 3), there was a difference in the degree of gene expression between the high and low toxic nanomaterials. It is considered that the decrease in the expression of chemokines may have proceeded from the cessation of inflammation at 1 week following intratracheal instillation. Oh JH et al reported that the expression of *CXCL5*, *CCL2* and *CCL7* in rat lungs from inhaled stainless-steel welding fumes returned to control levels earlier than inflammation as pathological changes in the lung [28].

Regarding the up-regulation of these 3 genes, there seems to be no common transcription factor, but it may be involved in the activation of TGF- β -activated kinase 1 (TAK1), which activates NF- κ B, JNK, and p38 MARK, known transcription factors and regulatory factors for many chemokines. Thiesfes et al. reported that the gene expression of *CXCL5*, *CCL2* and *CCL7* in the same experimental condition was stimulated by TNF through TAK1 activity in NIH3T3 cells [40]. Li et al. also reported that TAK1 inhibition suppressed inflammation and fibrosis in a pneumoconiosis animal model [41].

As described above, since the gene expression of the 3 chemokines is stable and reflects the difference in pulmonary toxicity of nanomaterials between different doses (0.8 and 4 mg/kg), and different observation periods (1 week and 1 month as observation times), these 3 chemokine genes, *CXCL5*, *CCL2* and *CCL7*, are considered to be useful as biomarkers for the ranking of the pulmonary toxicity of nanomaterials.

When the pulmonary toxicity of the nanomaterials was screened in this intratracheal instillation model, it is important that there were no false negatives. If the screening criteria for pulmonary toxicity of nanomaterials is that any of the 3 gene expressions exceed the cut-off value, false negatives as a screening test would have been minimum in this experiment (Table 3). Therefore, we think that the combination of these 3 genes, *CXCL5*, *CCL2* and *CCL7*, is useful for screening the pulmonary toxicity of nanomaterials.

Conclusion

We analyzed the mRNA expression of chemokines in rat lungs following intratracheal instillation of 4 different nanomaterials in order to find useful predictive markers of the pulmonary toxicity of nanomaterials. Our results suggest that 3 chemokine genes, especially *CXCL5*, followed by *CCL2* and *CCL7*, can be useful as biomarkers for the ranking of the pulmonary toxicity of nanomaterials.

Methods

Sample nanomaterials

We used nickel oxide (NiO), cerium dioxide (CeO₂), titanium dioxide (TiO₂), and zinc oxide (ZnO) as industrial nanomaterials in the present study. Commercially available NiO (US3355, US Research

Nanomaterials, Houston, TX), CeO₂ (Wako Chemical, Ltd. Japan), TiO₂ (Rutile) (MT-150AW, Teyca Co. Ltd., Osaka, Japan), and ZnO (Sigma-Aldrich Co. LLC., Tokyo, Japan) were dispersed in 0.4 ml distilled water. The physicochemical profiles of these samples are shown in Table 4 [21, 39, 42-44]. We defined the toxicity of the chemicals as follows: the chemicals which induced either sustained inflammation, fibrosis or tumors were set as having high pulmonary toxicity, and the chemicals that did not induce any of those pathological lesions were set as having low pulmonary toxicity. Accordingly, NiO and CeO₂ were classified as nanomaterials with high pulmonary toxicity [10, 21, 42], and TiO₂ and ZnO were classified as nanomaterials with low pulmonary toxicity [21, 39, 44, 45].

Animals

Male Fischer 344 rats (9–11 weeks old) used for exposure to nanomaterials were purchased from Charles River Laboratories International, Inc., Kanagawa, Japan. The animals were kept in the Laboratory Animal Research Center of the University of Occupational and Environmental Health for 2 weeks with free access to a commercial diet and water. All procedures and animal handling were done according to the guidelines described in the Japanese Guide for the Care and Use of Laboratory Animals as approved by the Animal Care and Use Committee, University of Occupational and Environmental Health, Japan.

Intratracheal instillation

The NiO, TiO₂, CeO₂, and ZnO nanomaterials were suspended in 0.4 ml distilled water. Doses of 0.2 mg (equivalent to 0.8 mg/kg BW) or 1 mg (equivalent to 4 mg/kg BW) were administered to rats (12 weeks old) in a single intratracheal instillation. Each of the negative control groups received distilled water.

Animals following intratracheal instillation

In the exposure to the 4 different nanomaterials and the control, there were 5 rats in each group at each time point. Animals were dissected at 3 days, 1 week, 1 month, 3 months and 6 months after intratracheal instillation and the lung was divided into right and left lungs. Analysis of cDNA microarray and qRT-PCR was performed with the homogenized third lobe of the right lung, and histopathological evaluation was performed with the left lung inflated and fixed by 4% paraformaldehyde or 10% formaldehyde.

Total RNA extraction

The third lobes of the right lungs (n=5 per group per time point) were homogenized using a QIAzol lysis reagent with a TissueRupotor (Qiagen, CA, USA). Total RNA from the homogenates was extracted using a miRNeasy Mini Kit (Qiagen, CA, USA) following the manufacturer's instructions. RNA was quantified using a NanoDrop 2000 spectrophotometer (Thermo Fisher Scientific Inc., MA, USA), and the quality of the samples was analyzed by a Bioanalyzer 2100 (Agilent Technologies, CA, USA).

Microarray analysis

We used a 3D-Gene Rat Oligo Chips 20K (version 1.1) (Toray Industries, Tokyo, Japan), which could mount 20,174 genes, for the DNA microarray analysis. Total RNA extracted from the lungs of the 5 rats in the NiO-high dose group was mixed in equal amounts to make one sample, and that was amplified by the use of an Amino Allyl MessageAmp II aRNA Amplification Kit (Ambion, Inc., CA, USA). The control group was treated in the same manner. The antisense RNA (aRNA) were labeled with Cy5, using Amersham Cy5 Mono-Reactive Dye (GE Healthcare, Buckinghamshire, UK), and the labeled aRNA were hybridized at 37°C for 16 hours. The hybridization was performed according to the supplier's protocols [46]. The chips were washed and dried, and then scanned in an ozone-free environment using a 3D-Gene Scanner 3000 (Toray Industries, Tokyo, Japan) and analyzed by use of 3D-Gene Extraction Software (Toray Industries, Tokyo, Japan). The digitalized fluorescent signals provided by the above-described software were regarded as the raw data. All of the normalized data were globally normalized per microarray, such that the median of the signal intensity was adjusted to 25. The function of the enhanced expression genes was analysed by Database for Annotation Visualization and Integrated Discovery 6.8 [47].

Validation of gene expression data using quantitative real-time polymerase chain reaction

Total RNA extracted from the lungs at each observation point in each group were transcribed into cDNA (High-Capacity cDNATM Kit, Life Technologies, Tokyo, Japan). qRT-PCR assays were performed using TaqMan (TaqMan Gene Expression Assays, Thermo Fisher Scientific Inc., MA, USA) according to the manufacturer's protocol. The Assays-on-Demand TaqMan probes and primer pairs were *CXCL5* (Assay ID Rn00573587_g1), *CCL2* (Assay ID Rn00580555_m1), *CCL7* (Assay ID Rn01467286_m1), *CXCL10* (Assay ID Rn00594648_m1), and *CXCL11* (Assay ID Rn00788261_g1). All experiments were performed in a StepOnePlusTM Real-Time PCR Systems (Life Technologies, Tokyo, Japan). All expression data were normalized to endogenous control β -actin expression (Assay ID Rn00667869_m1).

Histopathology and immunohistochemistry

The obtained lung tissue, which was inflated and fixed with 4% paraformaldehyde or 10% formaldehyde under a pressure of 25 cm water, was embedded in paraffin, sectioned at a thickness of 4 μ m, and then stained with hematoxylin and eosin (H&E). The slides were assessed for histological changes (H&E stain) by a board-certified pathologist (New Histo. Science Laboratory Co., Ltd., Tokyo, Japan). The severity of the histological changes in the lung in the control and nanoparticle-exposed rats was scored as none (0), minimal (0.5), mild (1), moderate (2) or severe (3).

Upregulation of *CXCL5*, *CCL2* and *CCL7* was evaluated by immunostaining with rabbit anti-mouse *CXCL5* polyclonal antibody (1:200 dilution, bs-2549R; Bioss Inc., Woburn, USA), goat anti-rat *CCL2* polyclonal antibody (1:200 dilution, sc-1785; Santa Cruz Biotechnologies, Inc., CA, USA), and goat anti-mouse *CCL7* polyclonal antibody (1:50 dilution, sc-21202; Santa Cruz Biotechnologies, Inc., CA, USA), respectively, using the lung tissue samples from the NiO-high dose group of 1 month after intratracheal instillation.

Statistical analysis

Statistical analysis was carried out using JMP^R Pro software (JMP Version 14.2.0, SAS Institute Inc., NC, USA). *P* values <0.05 were considered to be significant. Dunnett's tests were used appropriately to detect individual differences in the gene expression levels of each of the 5 chemokines between those exposed to the 4 nanomaterial samples and the controls. We assigned the toxicity of the exposure nanomaterials as being high or low according to the gene expression levels of each of the 5 chemokines of each sample (20 samples for both high and low toxicity at each time point), and analyzed the sensitivity and specificity for high toxicity at each time point to create the ROC curves and AUCs. Youden's Index was used to determine the cut-off value. Youden's Index was defined as follows: Youden's Index = sensitivity + specificity - 1, where the definitions of sensitivity and specificity are shown in Additional file 2 (Table S2) together with specific examples using a confusion matrix. In the evaluation using the combination of chemokine genes, the cases where the expression of at least one gene was equal to or higher than the cut-off value were defined as positive. Spearman's rank correlation coefficient was used to estimate the correlation between gene expression levels of *CXCL5*, *CCL2*, *CCL7*, *CXCL10* or *CXCL11* and the score of inflammatory cell infiltration of lung tissue.

Abbreviations

NiO: Nickel oxide; CeO₂: Cerium dioxide; TiO₂: Titanium dioxide; ZnO: Zinc oxide; *CXCL5*: C-X-C motif chemokine 5; *CCL2*: C-C motif chemokine 2; *CCL7*: C-C motif chemokine 7; *CXCL10*: C-X-C motif chemokine 10; *CXCL11*: C-X-C motif chemokine 11; ROC: Receiver operating characteristic; AUC: Area under the curves; POT fibers: potassium octatitanate fibers

Declarations

Ethics approval and consent to participate

All procedures and animal handling were performed in accordance with the guidelines set forth in the Japanese Guide for the Care and Use of Experimental Animals, approved by the Animal Care and Use Committee of the Japanese College of Occupational Medicine.

Consent for publication

Not applicable.

Availability of data and materials

The datasets used and/or analysed during the current study are available from the corresponding author on reasonable request.

Competing interests

The authors declare that they have no competing interests.

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Authors' contributions

Authors CN, HI, KY and YM are responsible for the study design and writing of the manuscript. Authors CN, HI, TT, JT and KY are responsible for data and analysis. Authors CN, HI, TT and YM performed the experiments. All the authors read and approved the final manuscript.

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Tables

Table 1.

(A) Number of genes by mRNA expression level in the NiO-high dose group at 1 month.

	mRNA level (fold change of control)	Number of genes
Up regulation	≥ 2 -fold	450
	2~4-fold	383
	4~8-fold	51
	≥ 8 -fold	16
Down regulation	$\leq 1/2$ -fold	388
	$1/2 \sim 1/4$ -fold	359
	$1/4 \sim 1/8$ -fold	15
	$\leq 1/8$ -fold	14

Table 1(A) shows the number of genes by mRNA expression level in the NiO-high dose group at 1 month after intratracheal instillation among 20,174 genes examined using cDNA microarray.

(B) Description of the genes related to 'inflammatory response' among the 16 genes upregulated ≥ 8 -fold.

Gene symbol	Gene description	Fold change
<i>CXCL5</i>	Chemokine (C-X-C motif) ligand5	12.83
<i>CCL2</i>	Chemokine (C-C motif) ligand2	17.93
<i>CCL7</i>	Chemokine (C-C motif) ligand7	23.44
<i>CXCL10</i>	Chemokine (C-X-C motif) ligand10	16.42
<i>CXCL11</i>	Chemokine (C-X-C motif) ligand11	16.70

There were 5 genes related to inflammation of the 16 genes in the analysis using Database for Annotation Visualization and Integrated Discovery 6.8 (DAVID 6.8, <https://david.ncifcrf.gov/>).

Table 2. ROC analysis between gene expression and pulmonary toxicity of nanomaterials.

Observation time	3 days	1 week	1 month	3 months	6 months
	AUC	AUC	AUC	AUC	AUC
	(95% C.I.)	(95% C.I.)	(95% C.I.)	(95% C.I.)	(95% C.I.)
	(Cut-off value)	(Cut-off value)	(Cut-off value)	(Cut-off value)	(Cut-off value)
<i>CXCL5</i>	0.990 (0.982-0.999) (10.008)	0.998 (0.961-1.000) (10.308)	0.995 (0.948-1.000) (9.513)	0.983 (0.874-0.998) (3.277)	0.928 (0.803-0.976) (1.934)
<i>CCL2</i>	0.693 (0.501-0.835) (11.495)	0.973 (0.841-0.996) (3.919)	0.980 (0.904-0.996) (1.945)	0.910 (0.753-0.971) (2.059)	0.848 (0.674-0.937) (1.076)
<i>CCL7</i>	0.680 (0.487-0.826) (12.380)	0.968 (0.860-0.993) (5.090)	0.993 (0.933-0.999) (1.990)	0.898 (0.744-0.963) (2.007)	0.893 (0.720-0.964) (1.020)
<i>CXCL10</i>	0.603 (0.415-0.764) (8.311)	0.795 (0.592-0.912) (2.088)	0.848 (0.673-0.974) (1.620)	0.778 (0.593-0.894) (1.466)	0.663 (0.473-0.811) (1.097)
<i>CXCL11</i>	0.525 (0.340-0.704) (7.843)	0.890 (0.744-0.958) (2.790)	0.883 (0.741-0.952) (2.073)	0.813 (0.612-0.922) (1.025)	0.920 (0.798-0.971) (0.706)

AUC: Area under the curve; 95% C.I.: 95% Confidence interval

Table 3. Sensitivity and specificity of gene expression of 5 chemokines in the pulmonary toxicity of nanomaterials.

		Sensitivity	Specificity	False positive	False negative
1 week	<i>CXCL5</i>	0.95 (19/20)	1.00 (20/20)	0.00 (0/20)	0.05 (1/20)
	<i>CCL2</i>	1.00 (20/20)	0.90 (18/20)	0.10 (2/20)	0.00 (0/20)
	<i>CCL7</i>	0.95 (19/20)	0.90 (18/20)	0.10 (2/20)	0.05 (1/20)
	<i>CXCL10</i>	0.75 (15/20)	0.90 (18/20)	0.10 (2/20)	0.25 (5/20)
	<i>CXCL11</i>	0.75 (15/20)	0.95 (19/20)	0.05 (1/20)	0.25 (5/20)
	<i>CXCL5+CCCL2+CCL7</i>	1.00 (20/20)	0.90 (18/20)	0.10 (2/20)	0.00 (0/20)
1 month	<i>CXCL5</i>	0.95 (19/20)	1.00 (20/20)	0.00 (0/20)	0.05 (1/20)
	<i>CCL2</i>	1.00 (20/20)	0.90 (18/20)	0.10 (2/20)	0.00 (0/20)
	<i>CCL7</i>	1.00 (20/20)	0.95 (19/20)	0.05 (1/20)	0.00 (0/20)
	<i>CXCL10</i>	0.65 (13/20)	1.00 (20/20)	0.00 (0/20)	0.35 (7/20)
	<i>CXCL11</i>	0.65 (13/20)	0.95 (19/20)	0.05 (1/20)	0.35 (7/20)
	<i>CXCL5+CCCL2+CCL7</i>	1.00 (20/20)	0.90 (18/20)	0.10 (2/20)	0.00 (0/20)

Sensitivity, Sensitivity (the number of true positives/the number of true positives + the number of false negatives)

Specificity, Specificity (the number of true negatives/the number of false positives + the number of true negatives)

False positive, False positive (the number of false positives/the number of false positives + the number of true negatives)

False negatives, False negatives (the number of false negatives/the number of true positives + the number of false negatives)

Table 4. Physiochemical characterization of the nanomaterials used in the present study.

	mRNA level (fold change of control)	Number of genes
Up regulation	≥ 2 -fold	450
	2~4-fold	383
	4~8-fold	51
	≥ 8 -fold	16
Down regulation	$\leq 1/2$ -fold	388
	$1/2 \sim 1/4$ -fold	359
	$1/4 \sim 1/8$ -fold	15
	$\leq 1/8$ -fold	14

Table 4 shows the property of each nanomaterial used in present study.

Figures

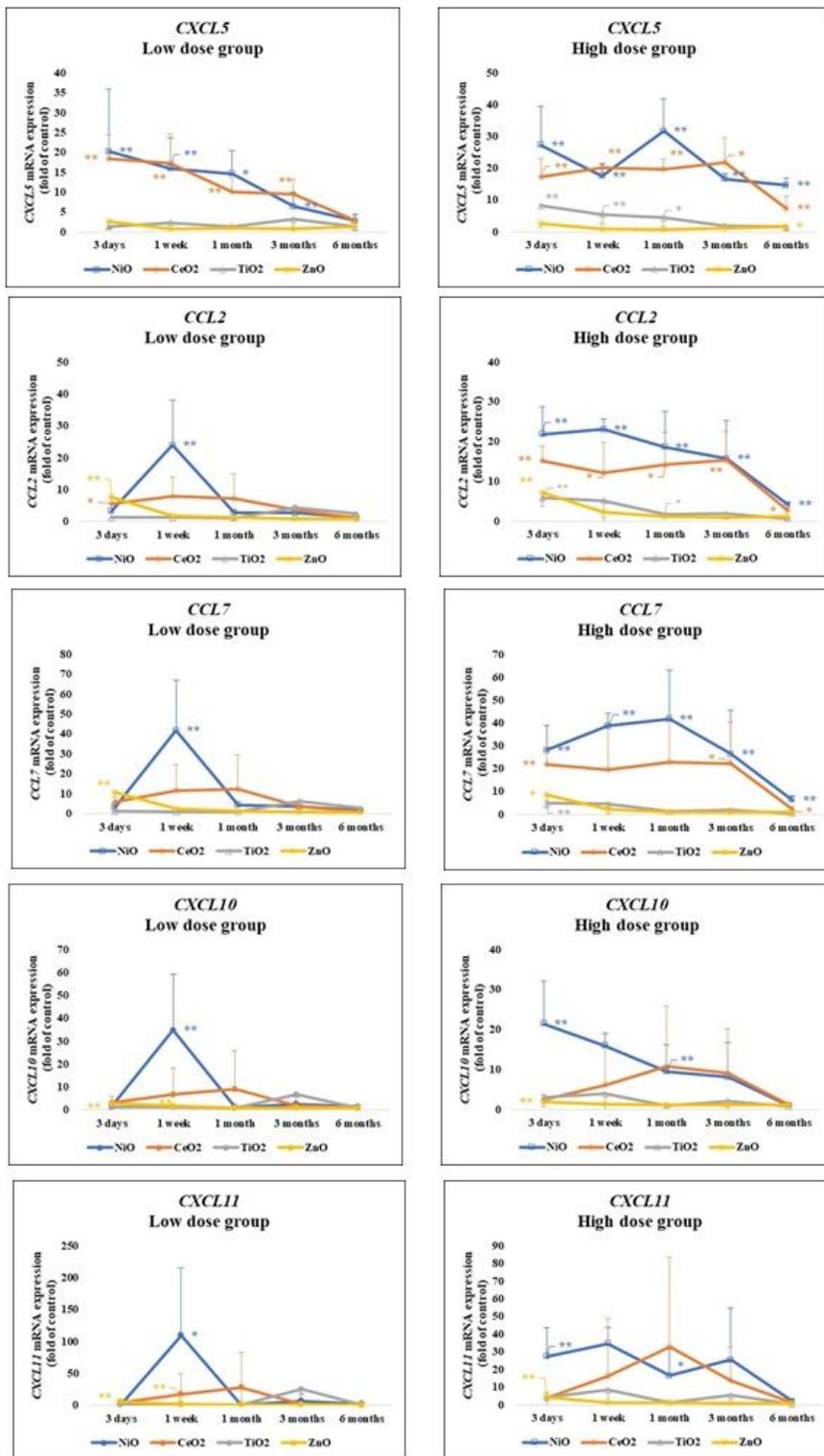


Figure 1

Gene expression of 5 chemokines in lung exposed to nanomaterials with different pulmonary toxicities. Values changes are mean \pm standard error (SE) ($p < 0.05$, $n=5$). Increased expression of CXCL5 gene in the NiO and CeO2 groups was persistently higher, and that in the TiO2 and ZnO groups transiently higher compared with the control groups, respectively. CCL2 and CCL7 also showed a similar tendency to CXCL5. (* $p < 0.05$, ** $p < 0.01$)

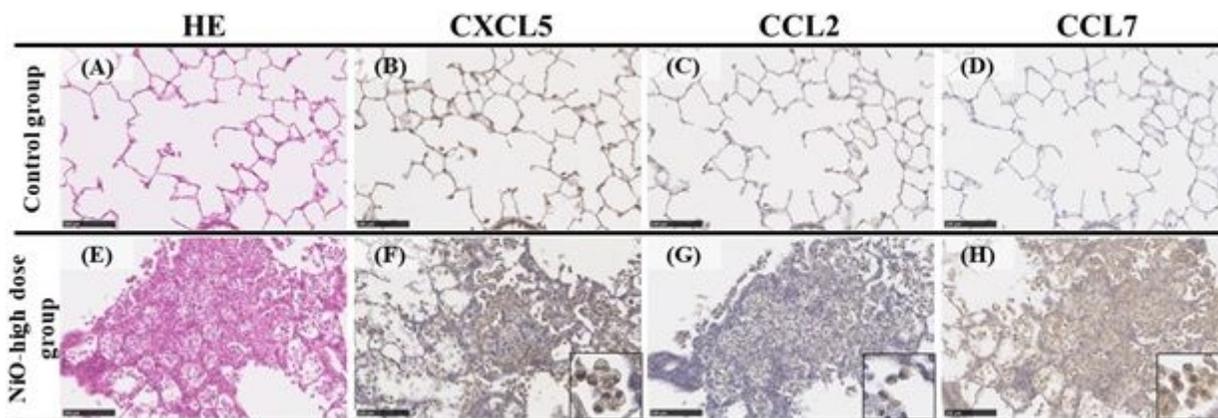


Figure 2

Representative images of CXCL5, CCL2, and CCL7 immunostaining in lung tissue exposed to NiO. Lung samples from the control and NiO-high dose exposed rats were sectioned with Hematoxylin staining ((A) and (E)) or CXCL5, CCL2 or CCL7 immunostaining with anti- CXCL5((B) and (F)), CCL2(C) and (G)) or CCL7((D) and (H)), respectively. All examples illustrate findings at 1 month after intratracheal instillation: Positive cells of CXCL5, CCL2 and CCL7 immunostaining on NiO-exposed lungs were mainly macrophages. (internal scale bar = 100 μm for all).

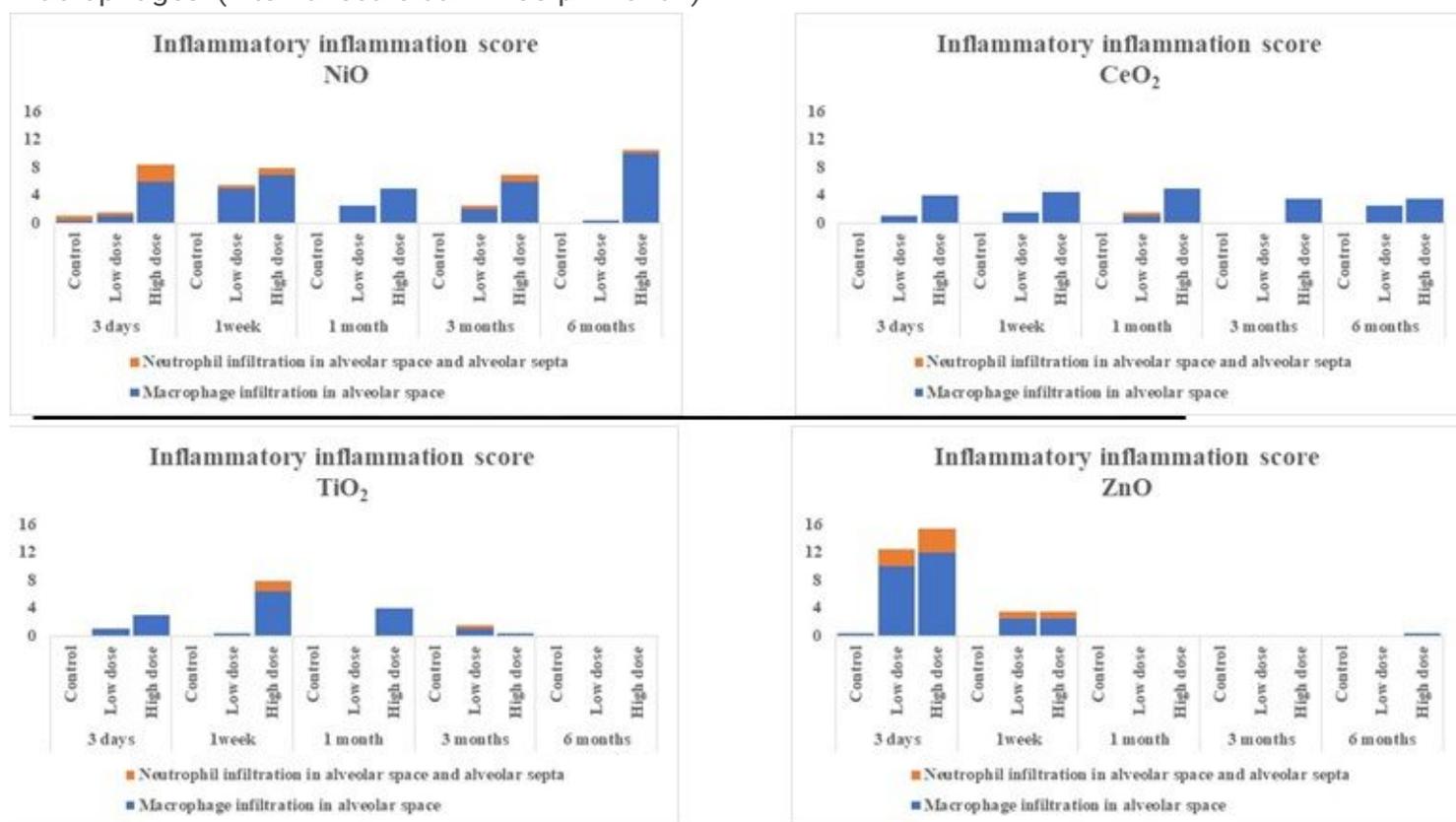


Figure 3

Score of inflammatory cell infiltration in the lung exposed to nanomaterials. The severity of lung histological changes in the control and nanoparticle-exposed rats was scored as none (0), minimal (0.5), mild (1), moderate (2), or severe (3). Exposure to NiO and CeO₂ resulted in persistent infiltration of inflammatory cells throughout the observation period, but TiO₂ and ZnO only showed a transient infiltration.

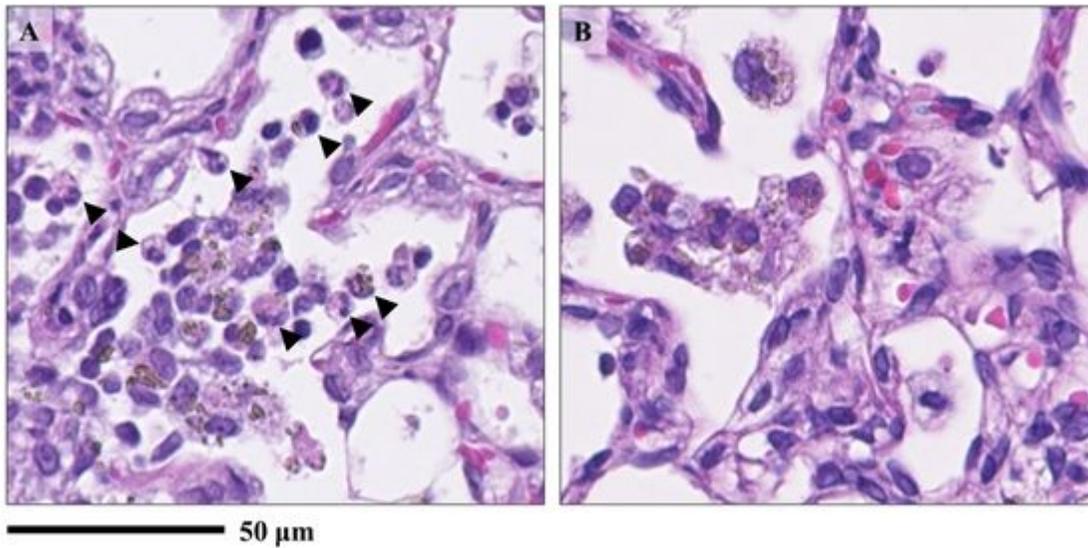


Figure 4

Lung samples sectioned with Hematoxylin staining exposed to nanomaterials intratracheally. There were differences of infiltrating inflammatory cells between nanomaterials. While mainly neutrophils and macrophages were found in the alveoli in the NiO-high dose group, macrophage-based inflammatory cell infiltration was observed in the CeO₂-high dose group. Arrow heads indicate neutrophils.

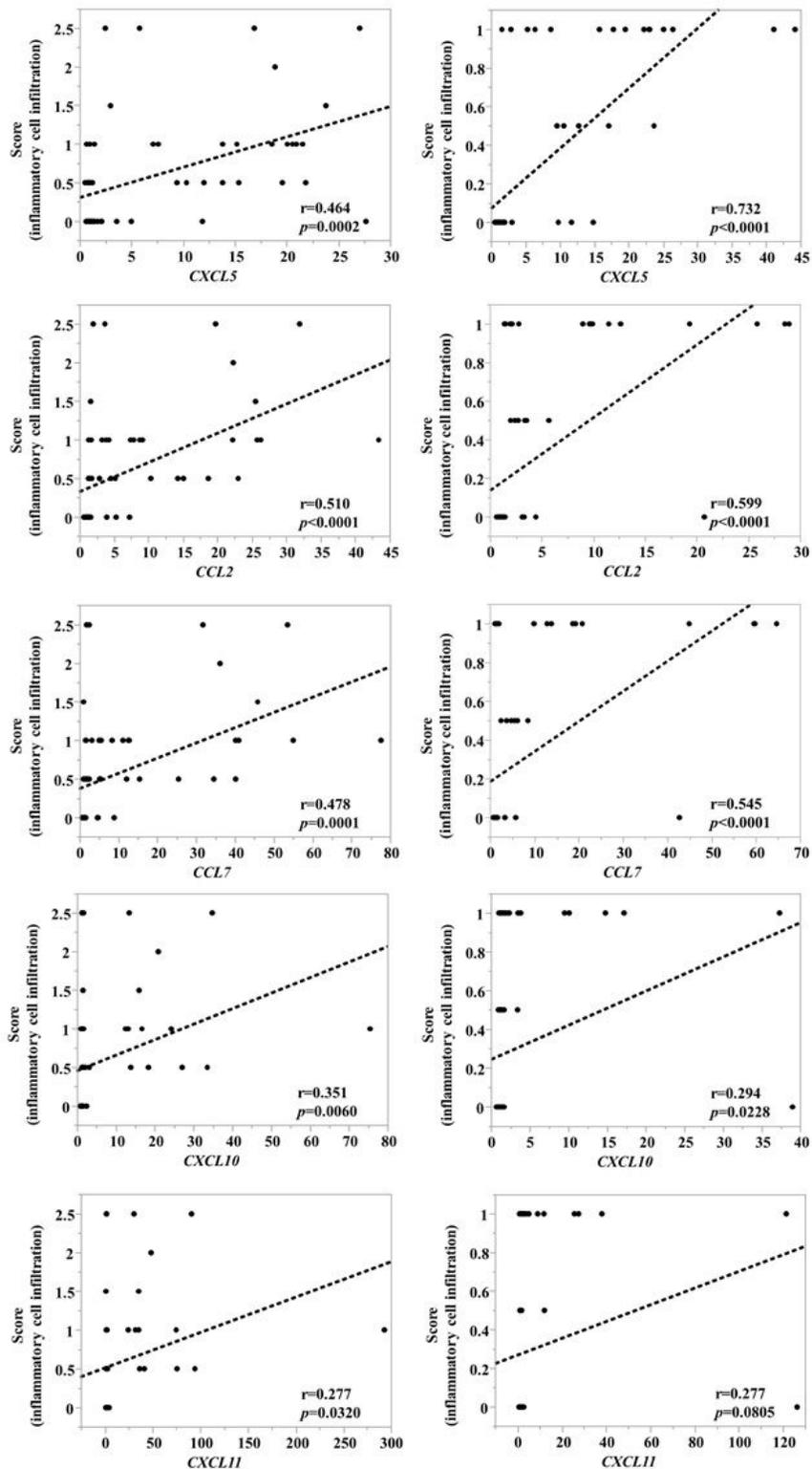


Figure 5

Relationship between inflammatory cell infiltration and gene expression of each 5 chemokines in exposed lung. There was relatively good correlation between inflammatory cell infiltration in lung tissues and CXCL5, CCL2, and CCL7 at 1 week and 1 month after intratracheal instillation.

Supplementary Files

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