(2) avoiding social situations, such as attending school or going to a workplace (duration of at least 6 months); (3) avoiding social relationships, such as friendships or contact with family members (duration of at least 6 months); and (4) significant distress or impairment due to social isolation.

Self-report measures. We administered the University of California, Los Angeles (UCLA) Loneliness Scale, the Lubben Social Network Scale-6(LSNS-6), the Sheehan Disability Scale (SDS), the Cornell Treatment Preferences Index (CTPI) and a questionnaire on sociodemographic characteristics to participants.

The UCLA Loneliness Scale is a 20-item questionnaire that assesses how often individuals endorse subjective feelings of loneliness (e.g. 'How often do you feel that you lack companionship?'). The score range is 20 to 80, with higher scores indicating greater degrees of loneliness (Russell, 1996). Each item is rated on a 4-point scale from 1 ('never') to 4 ('always'). As the Revised UCLA Loneliness Scale has been validated Korean and Japanese samples, it was used at these sites (Kim, 1997; Kudou & Nishikawa, 1983). At the United States and Indian sites, Version 3 of the UCLA Loneliness Scale was used. Version 3 is identical to the revised version, except for minor wording adjustments (Russell, 1996).

The LSNS-6 is a 6-item questionnaire that assesses the number of people in an individual's social network with whom one has social contact (e.g. 'How many relatives do you see or hear from at least once a month?') and who are a source of social support (e.g. 'How many friends do you feel close to such that you could call on them for help?'). There are two subscales for family and friends. The total score range is 0–30 (0–15 for each subscale), and a total score less than 12 is indicative of social isolation (Lubben et al., 2006). Such a score implies fewer than two social network members, on average, for each item. Each item is rated on a 6-point scale from 0 ('none') to 5 ('nine or more'). The LSNS-6 has been validated in Korean and Japanese (Hong, Casado, & Harrington, 2011; Kurimoto et al., 2011).

The SDS is a 5-item questionnaire that assesses disability or functional impairment. The first three items evaluate level of disruption in each of three domains (work/school, social life and family life/home responsibilities) with response choices on a 0 ('not at all') to 10 ('extremely') scale, while the remaining two items evaluate days lost and days unproductive (Sheehan, 1983). Higher scores indicate more disability. The word 'symptom' in the SDS was replaced with 'social isolation' for this study. The scale has been validated in Korean and Japanese (Lee & Song, 1991; Yoshida, Otsubo, Tsuchida, Wada, & Kamijima, 2004).

The CTPI is a 6-item questionnaire that evaluates several different depression treatment preferences, including treatment modality and type of treatment provider (Raue, Schulberg, Heo, Klimstra, & Bruce, 2009). We modified the CTPI to assess preferences related to social isolation (e.g. 'I wish to receive counseling or psychotherapy for my social isolation'). The response scale for the first five items is a 5-point Likert scale from 1 ('strongly disagree') to 5 ('strongly agree'), and the final item uses ranked treatment preferences. For the CTPI, as well as other instruments that lacked an existing translation in a target language, we translated the instrument and used back translation as verification of adequate adaption.

Statistical analysis

We compared variables using the t-test and chi-square for continuous and categorical variables, respectively. When any group or cell contained five or fewer participants, we replaced the t-test and chi-square with the Wilcoxon Rank-Sum test and Fisher's exact test, respectively. Linear regression models were used to examine the association between country and several outcome variables, including loneliness, social network and functional disability. Logistic regression models were similarly used for the association between country and the dichotomized treatment preferences. The regression models were adjusted for the effects of the educational level and age as these were significant in bivariate correlations with country. Sample sizes for particular analyses vary due to differences in number of responses. Significance level for all tests was set at $p < .05$ and tests were two-tailed. Data were analyzed using Stata Version 12 (Stata Corp.)

Results

Identification of hikikomori

Regarding the first aim, 36 adult participants with social withdrawal who met criteria for hikikomori were identified. The cases were found in all four countries included in this study. As seen in Table 1, the vast majority were men with varied education levels. The majority of participants lived with family members; just four (11%) lived alone. Their self-reported period of social withdrawal was on average 2.1 years.

Psychosocial features

We quantitatively described a number of features of individuals with hikikomori. Scores on the UCLA Loneliness Scale indicated a high level of loneliness among all participants ($M = 55.4$, $SD = 10.5$). By comparison, prior studies with normal controls in American, Indian and Korean samples have shown mean scores of about 40 (SD around 9) (Jayashankar, 2013; Lee & Lee, 2004; Russell, 1996), and studies with depressed participants have shown average scores of 49.8 (Grov, Golub, Parsons, Brennan, & Karpiak, 2010). Likewise, social networks for our sample were

Table 1. Sociodemographic characteristics of participants with hikikomori in four countries.

Characteristic	Total	Japan	USA	India	Korea		p
	(*n* = 36)	(*n* = 11)	(*n* = 11)	(*n* = 10)	(*n* = 4)		
	n (%)	*n* (%)	*n* (%)	*n* (%)	*n* (%)		
Male	29 (81)	10 (91)	7 (64)	9 (90)	3 (75)		.33
Age (years)							
18–21	11 (32)	2 (18)	2 (18)	3 (30)	4 (100)	}	.04
22–30	11 (32)	3 (27)	4 (36)	6 (60)	0 (0)		
31–49	12 (35)	6 (55)	5 (45)	1 (10)	0 (0)		
Education level							
High school graduate or less	16 (44)	7 (64)	2 (18)	3 (30)	4 (100)	}	.01
Some college or more	20 (56)	4 (36)	9 (81)	7 (70)	0 (0)		
Living situation							
Lives with others	32 (89)	10 (91)	8 (73)	10 (100)	4 (100)	}	.2
Lives with no one	4 (11)	1 (9)	3 (27)	0 (0)	0 (0)		

weak, with participants scoring a mean of 9.7 (SD = 5.7) on the LSNS-6. By comparison, prior studies with normal controls have shown average scores of 17.4 (Lubben et al., 2006). Individuals with hikikomori showed slightly higher scores on the family subscale (M = 5.4, SD = 3.0) than the friend subscale (M = 4.3, SD = 3.5). Participants with hikikomori had moderate levels of functional disability on the SDS (M = 16.5, SD = 7.9), levels comparable to patients with psychiatric disorders and more than three-fold higher than those with no mental illness in a study of a study of primary care patients (Olfson et al., 1997). Impairment was highest in terms of social life/leisure activities, compared to work/school and family life.

Treatment preferences

A total of 78% of the sample expressed a desire for treatment for their social withdrawal. In terms of modality of treatment, participants preferred psychotherapy (M = 3.6, SD = 1.5) over medication (M = 2.9, SD = 1.4); $t(31) = 2.13$, p = .04. In addition, participants also were significantly more likely to be interested in psychotherapy and medicine management delivered *in-person* compared to an option for provision by *webcam* (p < .001 for both comparisons). Participants ranked individual psychotherapy most as a desired treatment, with few desiring complementary and alternative treatments such as herbal remedies or exercise (Figure 1). As for treatment provider, participants preferred mental health specialists (M = 3.6, SD = 1.2) over primary care physicians (M = 2.7, SD = 1.2); $t(34) = 3.87$, $p < .001$.

Cross-national comparisons

We compared treatment preferences and psychosocial characteristics of participants across the four countries in this study as our exploratory aim: that is, to generate hypotheses about cross-national differences in hikikomori that might be tested in future studies. Across countries, results generally were similar. For comparison of treatment preferences across countries, the Korean sample was excluded from analyses due to small sample size (n = 4). In adjusted models controlling for age and level of education, there were no statistically significant differences in overall desire for treatment, desire for pharmacotherapy, desire for psychotherapy, interest in webcam-delivered medication management or psychotherapy, interest in in-person-delivered medication management or desire for treatment provided by a mental health professional. Participants in the United States were significantly less likely to desire treatment by a primary care physician compared to Japan (odds ratio (OR) = 0.04, 95% confidence interval (CI) = 0.00–0.60). Also, Indian participants had a significantly lower interest in in-person psychotherapy (OR = 0.00, 95% CI = 0.00–0.31). Table 2 illustrates psychosocial features of our sample of individuals with hikikomori. As illustrated by the beta coefficient, American participants demonstrated on average a 12-point higher score on the UCLA Loneliness Scale and a 4-point higher score on the family life subscale of the SDS, as compared with Japanese participants. Indian participants had significantly stronger social networks but higher levels of functional disability. Finally, Korean subjects had significantly higher levels of loneliness, weaker friendships in their social network and higher functional disability.

Discussion

This study bolsters evidence that hikikomori, as a phenotype of severe social withdrawal, exists cross-nationally. Strengths of our approach include use of a

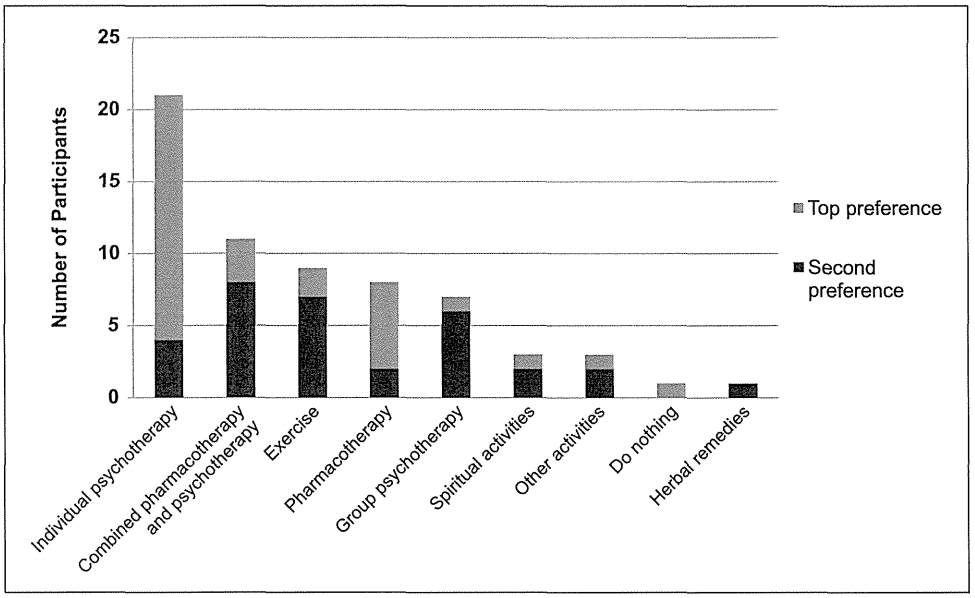

Figure 1. Top two treatment preferences of participants with hikikomori for their social withdrawal (n = 32).

Table 2. Multivariable linear regression of exploratory associations between psychosocial features of hikikomori and country.

	Japan	USA		India		Korea	
	(n = 11)	(n = 11)		(n = 10)		(n = 4)	
Characteristic	β	β	(95% CI)	β	(95% CI)	β	(95% CI)
Loneliness (UCLA Loneliness Scale)	Ref	12.35**	(5.41, 19.29)	−3.78	(−10.90, 3.33)	16.31**	(6.44, 26.17)
Social network (Lubben Social Network Scale – 6)	Ref	0	(−4.69, 4.68)	5.05*	(0.24, 9.85)	−5.37	(−12.03, 1.29)
Family subscale	Ref	−0.24	(−2.77, 2.30)	3.41*	(0.81, 6.01)	−0.86	(−4.46, 2.75)
Friend subscale	Ref	0.23	(−2.73, 3.19)	1.64	(−1.40, 4.67)	−4.51*	(−8.72, −0.31)
Functional disability (Sheehan Disability Scale)	Ref	4.95	(−1.90, 11.81)	9.04*	(2.16, 15.92)	13.86*	(3.44, 24.27)
Disrupted work/school work	Ref	−0.36	(−3.40, 2.68)	2.20	(−0.85, 5.25)	1.49	(−3.13, 6.12)
Disrupted social life/leisure activities	Ref	2.04	(−0.29, 4.36)	2.86*	(0.48, 5.24)	4.67*	(1.01, 8.32)
Disrupted family life/home responsibilities	Ref	4.03**	(1.54, 6.52)	4.06**	(1.51, 6.61)	7.70***	(3.78, 11.61)

UCLA: University of California, Los Angeles.
Analyses controlled for age and level of education. Japan used as the reference group (Ref) for country comparisons.
*statistically significant at the .05 level; **statistically significant at the .01 level; ***statistically significant at the .001 level.

standardized definition and assessment tool for hikikomori across four countries with diverse cultures and operationalizing hikikomori with discrete questions about the frequency, length and quality of social withdrawal. Past approaches have relied on a single, complex question (Koyama et al., 2010; Umeda, Kawakami, & The World Mental Health Japan Survey Group, 2002–2006, 2012), an approach that may cause misunderstanding by placing a high cognitive burden on the respondent (Schwarz, 2007). Thus, this study offers a new interview tool to help assess for hikikomori. Our data showing loneliness and limited connections with social network members among study participants support the validity of our assessment approach to hikikomori as we have defined it.

Psychosocial features

Perhaps the most striking features of hikikomori participants in this study were high loneliness scores and impaired social network scores. Our descriptive data paint a picture of the average individual with hikikomori being intensely lonely and deficient in social support, apparently unable to maintain meaningful social ties. This is despite rarely living alone and indicating a desire for treatment of their social withdrawal.

Treatment preferences

In these individuals who have been avoided social contact for such a prolonged period of time, we were surprised to find a consistent preference for treatment delivered in-person, as opposed to telepsychiatry-style. We believe this is the first study to describe treatment preferences in a sample of individuals with hikikomori. Understanding treatment preferences is a valuable first step for intervention research, particularly in light of evidence that treatment response rates for hikikomori are low (Nagata et al., 2013). Individuals with hikikomori may feel ambivalent about their desire for social relationships, and a patient–provider relationship may offer an entry point into re-establishing social connections. Given these results, future intervention studies for hikikomori might consider evaluating home visitation, particularly when conducted by a mental health professional and with an aim of boosting the social support of hikikomori patients (Dickens, Richards, Greaves, & Campbell, 2011; Lee et al., 2013). Other interventions that have shown promise in populations with mental illnesses and are thought to work by bolstering social relationships, such as peer support, might be investigated (Pfeiffer, Heisler, Piette, Rogers, & Valenstein, 2011; Proudfoot et al., 2012).

Limitations

This study was designed as a case series, and therefore several limitations in interpretation of the results bear note. First, our sample was small, but we have employed statistical methods that adjust for sample size. Second, cross-national comparisons should only be regarded as exploratory because different recruitment methods were used across countries, data harmonization across cultures is always imperfect and adjustment for potential confounders was limited to basic sociodemographic variables. Third, individuals with hikikomori who are able to participate in a research study such as this are unlikely to be representative of all of those with hikikomori. In particular, individuals with hikikomori are often perceived as resistant to undergo treatment, and our sample may represent those who have milder symptoms or begun recovery. Nonetheless, this highlights a group that may represent great opportunity for intervention. Fourth, as this was primarily a descriptive study, no comparison group was included, though we have included comparisons with normative data for selected measures. Finally, the CTPI has not been validated in international samples, and therefore treatment preference data must be interpreted cautiously.

Conclusion

In sum, this study suggests that hikikomori exists cross-nationally, can be assessed with a brief interview tool and is associated with substantial loneliness, impaired social networks, disability and desire for treatment. Results of our study suggest several possible directions for future research. First, we believe future cross-national studies of hikikomori should obtain larger samples, which could be achieved by focusing on just two locations for comparison. Another approach would be to compare hikikomori participants to a control group such as participants with social anxiety disorder to help tease out differences between hikikomori and other conditions. Although it was beyond the scope of this study to conduct formal psychometric testing on our hikikomori assessment tool, future research on the reliability and validity of the hikikomori diagnostic interview would be helpful. Furthermore, development and testing of a hikikomori scale could help with conceptual clarity (e.g. constructs associated with hikikomori) and distinction from related conditions such as social anxiety disorder. Once validated, a hikikomori scale or diagnostic interview could then be applied to research on the prevalence and detection of hikikomori. To reach a more representative sample including individuals unable to leave their residence under any circumstance, Internet-based surveys on hikikomori should be developed. Finally, interventions that account for patient preference might be tested.

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Conflict of interest

The authors declare that there is no conflict of interest.

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Appendix 1

esdate **Date of Screening:** _____ _____ _____
Month Day Year

Step 1: Give explanation of study (suggested script below)
'First, I will give you a little background information. This is a research study about social isolation. The purpose of this study is to find out how common social isolation is and difficulties are associated with it. In order to participate in the study, all interested individuals must participate in an interview and complete several questionnaires. No treatment will be provided as part of the study. There is compensation of $50 for time and costs to you. Your participation is completely voluntary. Whether you choose to participate or not does not have any effect on the medical services you receive. Next, I will ask you some background questions.'

Step 2: Collect demographic information

es1 Gender: $Female_0$ $Male_1$

es2 Ethnicity *(circle all that apply)*: $Asian_1$ $Black_2$ $Hispanic_3$ $White_4$ $Other_5$

es3 Age: ___ ___ **[If age is <18 or >39 years, mark 'Ineligible' in Step 6]**

Step 3: Collect contact information (on separate document)

Step 4: Review inclusion criteria

es4 1. Do you currently spend most of the day and nearly every day at home?
_____Yes_1 _____No_0

es5 *a (If yes)* When did it start?
_____ _____ _____
Month Day Year

es6 2. Have you ever in the past spent most of the day and nearly every day at home?
_____Yes_1 _____No_0

es7,es8 *a (If yes)* When did the longest past period start and end?
Start: _____ _____ _____ End: _____ _____ _____
Month Day Year Month Day Year

STOP AND ASSESS:
[If 1 is 'NO' and 2 is 'NO' mark 'Ineligible' in Step 6. If 1(a) is less than 6 months ago and period in 2(a) is less than 6 months, mark 'Ineligible' in Step 6]

es9 3. Do you currently avoid social situations, such as attending school or going to a workplace?
_____Yes_1 _____No_0

a (If yes) What are a couple (two) examples?
_____
_____
_____

es10 *b (If yes)* When did it start?
_____ _____ _____
Month Day Year

es11 4 Have you ever in the past avoided social situations?
_____Yes_1 _____No_0

es12,es13 *a (If yes)* When did the longest past period start and end?
Start: _____ _____ _____ End: _____ _____ _____
Month Day Year Month Day Year

STOP AND ASSESS:
[If 3 is 'NO' and 4 is 'NO', mark 'Ineligible' in Step 6. If 3(a) is less than 6 months ago and period in 4(a) is less than 6 months, mark 'Ineligible' in Step 6]

es14 5 Do you currently avoid social relationships, such as friendships or contact with family members?
_____Yes_1 _____No_0

a (If yes) What are a couple (two) examples?
_____
_____
_____

Appendix 1 (Continued)

es15 b *(If yes)* When did it start?

_____ _____ _____
Month Day Year

es16 6 Have you ever in the past avoided social relationships?
_____Yes$_1$ _____No$_0$

es17,es18 a *(If yes)* When did the longest past period start and end?
Start: _____ _____ _____ End: _____ _____ _____
Month Day Year Month Day Year

STOP AND ASSESS:
[If 5 is 'NO' and 6 is 'NO' mark 'Ineligible' in Step 6. If 5(a) is less than 6 months ago and period in 6(a) is less than 6 months, mark 'Ineligible' in Step 6]

es19 7 Considering your most severe period of social isolation, *(did/does)* it do any of the following:
a) interfere significantly with your normal routine;
b) interfere significantly with your ability to work or attend school;
c) interfere significantly with social activities or relationships; or
d) bother you a lot?
_____Yes$_1$ _____No$_0$
[If NO, mark 'Ineligible' in Step 6]

8 Briefly, what is/was the reason you started being socially isolated?
_____
_____
_____
[If all episodes due to a chronic physical illness or injury, mark 'Ineligible' in Step 6]

Step 5: Review of exclusion criteria

es20 1. Are you comfortable speaking and reading English*?
_____Yes$_1$ _____No$_0$ **[If NO, mark 'Ineligible' in Step 6]**

es21 2. Do you live in a group home or institution?
_____Yes$_1$ _____No$_0$ **[If YES, mark 'Ineligible' in Step 6]**

3. Do you have a history or have you been told you have any of the following conditions:
es22a _____ Schizophrenia
es22b _____ Dementia (any type)
es22c _____ Mental Retardation
es22d _____ Asperger Syndrome
es22e _____ Autistic Disorder (Autism)
[If any of the above conditions checked, mark 'Ineligible' in Step 6]

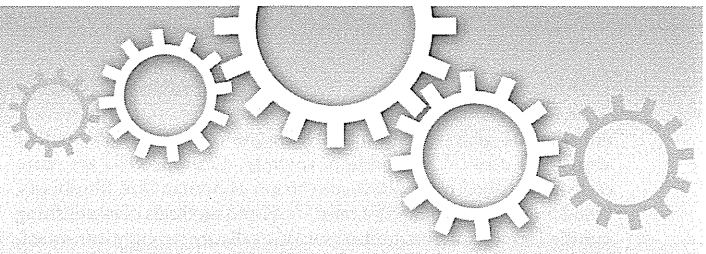
Step 6: Determine eligibility screening outcome

es23 1. _____$_0$ Ineligible for study participation.
'I'm sorry to say that you are not eligible to participate in this study. Thank you for your interest. Do you have any questions or concerns?'

2. _____$_1$ Eligible for study participation.
'I'm pleased to say that you are eligible to participate in this study.'

*English replaced by the local language for non-English-speaking sites.

SCIENTIFIC REPORTS



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SUBJECT AREAS:
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Direct induction of ramified microglia-like cells from human monocytes: Dynamic microglial dysfunction in Nasu-Hakola disease

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Microglia have been implicated in various neurological and psychiatric disorders in rodent and human postmortem studies. However, the dynamic actions of microglia in the living human brain have not been clarified due to a lack of studies dealing with *in situ* microglia. Herein, we present a novel technique for developing induced microglia-like (iMG) cells from human peripheral blood cells. An optimized cocktail of cytokines, GM-CSF and IL-34, converted human monocytes into iMG cells within 14 days. The iMG cells have microglial characterizations; expressing markers, forming a ramified morphology, and phagocytic activity with various cytokine releases. To confirm clinical utilities, we developed iMG cells from a patient of Nasu-Hakola disease (NHD), which is suggested to be directly caused by microglial dysfunction, and observed that these cells from NHD express delayed but stronger inflammatory responses compared with those from the healthy control. Altogether, the iMG-technique promises to elucidate unresolved aspects of human microglia in various brain disorders.

Microglia, immune cells in the brain, play major immunological/inflammatory roles as brain macrophage in the central nervous system (CNS). The origin of resident microglia have been proven to be from primitive myeloid progenitors (primitive macrophage) that arise in the yolk sac before embryonic day 8[1]. Resident microglia form as a ramified type (called ramified microglia), whose branches constantly move and survey the microenvironment under physiological conditions in the CNS[2], and once activated, shift to an ameboid type, phagocytose, and release various mediators such as pro-inflammatory cytokines[3–5]. Microglia are suggested to contribute to the pathophysiology of various neurological and psychiatric disorders[6–8]. Nasu–Hakola disease (NHD) which is a very rare autosomal recessive disorder, initially reported in Finland and Japan[9,10], is believed to be caused by microglial dysfunction. Until now, only about 200 cases have been reported worldwide and the majority of cases are in the Finnish and Japanese populations[11]. NHD is characterized by formation of multifocal bone cysts and progressive early-onset dementia with various psychiatric symptoms including personality changes[11,12], caused by mutations of DNAX-activation protein 12 (DAP12)[13] or triggering receptor expressed on myeloid cells 2 (TREM2)[14], both of which are expressed in human microglia. A rodent brain study showed that DAP12 is expressed only in microglia and deletion of DAP12 induces synaptic impairments possibly due to microglial dysfunction[15]. A human postmortem study has revealed the absence of DAP12 expression on ramified microglia in the brains of NHD patients[16].

The above-mentioned reports have strongly supported the theory that human microglia maladaptively contribute to a variety of neurological and psychiatric disorders including NHD, while dynamic analysis of microglial dysfunction in the human brain has yet to been clarified. The most significant limitation in human brain research is the difficulty in obtaining living brain cells including microglial cells from living human brains due to ethical and technical considerations. To solve this limitation, alternative methods have long been warranted. Presently, human neuronal cells can be established from somatic cells (not from the brain) such as skin fibroblasts by

utilizing the gene-modification technique of induced pluripotent stem (iPS) cells[17,18]. In addition, recently, neuronal cells are more easily established from direct conversion of human skin fibroblasts, called induced neuronal (iN) cells[19–21]. Novel methods of establishing ramified microglia from human somatic cells are strongly warranted, based on iPS or direct conversion techniques, while none have yet been reported. Herein, we show a novel technique for developing induced microglia-like (iMG) cells easily and quickly from adult human peripheral blood cells. In addition, by utilizing this iMG-technique, we present the first translational analysis of the dynamic actions of microglia from a patient of NHD.

Results

Inducing ramified microglia-like cells. To determine what cytokines induce ramified microglia from human peripheral monocytes, we selected and tested the effects of the following candidate cytokines; granulocyte-macrophage colony-stimulating factor (GM-CSF), macrophage colony-stimulating factor (M-CSF) and interleukin (IL) -34, all of which are suggested to be essential for developing and maintaining ramified microglia[22–25]. Untreated monocytes showed round shapes (Fig. 1A). Macrophages, induced by GM-CSF (10 ng/ml), shifted to an ameboid morphology on DAY 14 (Fig. 1B). On the other hand, treatment of M-CSF (10 ng/ml) alone or IL-34 (100 ng/ml) alone showed a spindle morphology (Fig. 1C and D), and the cocktail of both cytokines induced more complicated morphologies than the single treatment (Fig. 1E).

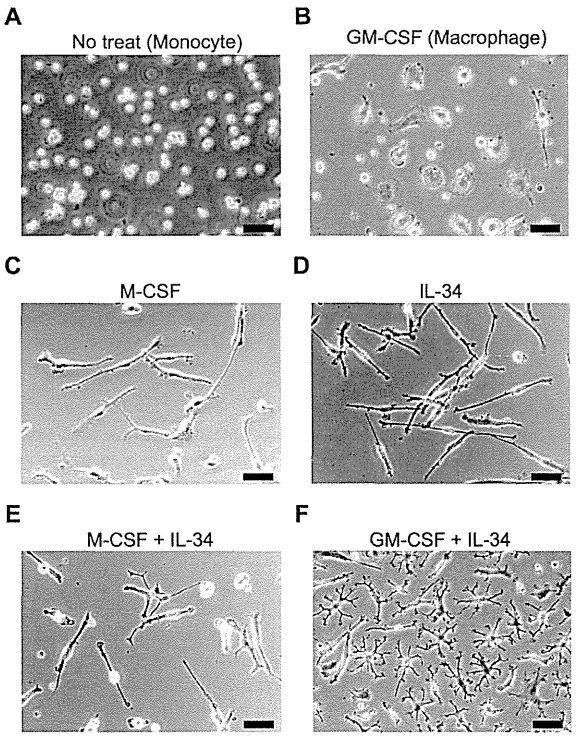

Figure 1 | Inducing ramified microglia from human peripheral monocytes. The monocytes on the day of isolation (A) were incubated with the following candidate cytokines; GM-CSF (10 ng/ml; B), M-CSF (10 ng/ml; C), IL-34 (100 ng/ml; D), M-CSF + IL-34 (E) and GM-CSF + IL-34 (F) for 14 days. The optimal cytokine conditions were tested by morphological changes using phase-contrast microscopy. The cocktail of both GM-CSF and IL-34 induced small soma body bearing numerous branched collaterals, which expressed the specific morphology of ramified microglia (F). Scale bar, 50 μm.

Surprisingly, the cocktail of both GM-CSF (10 ng/ml) and IL-34 (100 ng/ml) induced small soma bodies bearing numerous branched collaterals (Fig. 1F), which expressed the specific morphology of ramified microglia- small soma with extensive radial ramifications. The viability of these cells post 14 days was 16.7% ± 4.2 (n = 3, mean ± SEM) as compared to the initial cell number (DAY 0). Interestingly, the earliest branched cells were observed on DAY 3 after GM-CSF and IL-34 treatment (Supplementary Fig. S1A). In addition, we confirmed that these cells survive at least one month when medium change was performed once a week (Supplementary Fig. S1B).

Phenotyping of the induced microglia-like (iMG) cells. Next, we tested whether the ramified microglia-like cells, named *induced microglia-like (iMG) cells*, have microglial characterization. Generally, it is difficult to distinguish between macrophage and microglia, because useful and specific microglial markers are very limited. Traditionally, CD11b and CD45 are used as a distinction marker between macrophage and microglia[26]. Recently, the phenotype of human microglial cells, isolated from the fresh postmortem brain, has been revealed to have lower expression of CD14 and CD200R compared to macrophage[27]. Thus, we compared the expression level of surface markers between iMG cells and induced macrophage using flow cytometry. The expression level of CD11b on iMG cells did not differ from that on macrophage, while that of CD45 decreased on iMG cells (Fig. 2A). The expression levels of CD14 and CD200R were also decreased on iMG cells compared to those on macrophage (Fig. 2B), which support that iMG cells have the specific phenotype of microglia[27]. Furthermore, Mizutani et al.[28] recently reported a clear-cut distinction between monocytes ($CCR2^{high}$, $CX3CR1^{low}$) and resident microglia ($CCR2^{low}$, $CX3CR1^{high}$) using $CX3CR1^{+/GFP}CCR2^{+/RFP}$ knockin fluorescent protein reporter mice. Therefore, we compared the expression pattern of CCR2 and CX3CR1 between monocytes and iMG cells. Monocytes were stained with bright red fluorescence (CCR2) bearing round or elliptic morphology (Fig. 2C), and iMG cells were stained with bright green fluorescence (CX3CR1) bearing highly branched forms (Fig. 2, C and D). In addition, we confirmed that the expression ratio (CX3CR1/CCR2) of iMG cells is significantly higher than that of monocytes by flow cytometry (Fig. 2E). These results indicate that the iMG cells induced by GM-CSF and IL-34 show the essential characteristics of resident microglia[28].

Melief et al.[27] have also revealed that IL-4 and dexamethasone alter specific gene expressions in fresh human microglial cells ([IL-4] HLA-DR, CCR7, CCL18, and CD200R are upregulated, and CD45 and TNF-α are downregulated; [dexamethasone] CCL18 is upregulated, and HLA-DR, CCR7, CD45, TNF-α, and CD200R are downregulated). Therefore, we assessed the above gene expression patterns in iMG cells incubated with IL-4 and dexamethasone using quantitative reverse transcriptase-polymerase chain reaction (qRT-PCR). Except for CCL18 (treated with dexamethasone), almost all gene expression patterns (HLA-DR, CCR7, CD200R, CD45 and TNF-α) in the iMG cells were in agreement with the above data using fresh human microglia[27] (Fig. 2F, G).

Functional analysis of the iMG cells. Microglia reside as a ramified form, and various molecules activate microglia into an ameboid form, phagocytizing and releasing various cytokines[3], and overactivation of microglia induces neuronal damage and various brain pathologies via pro-inflammatory cytokines such as tumor necrosis factor (TNF) -α[6,7]. To examine whether iMG cells have these dynamic functions, we tested the phagocytosis ability and the following TNF-α secretion. Interestingly, the iMG cells showed the ability of phagocytosis with morphological changes into an ameboid form (Fig. 3A). Next, we tested the ability for TNF-α production during phagocytosis on the iMG cells, and revealed that the mRNA expression and protein level of TNF-α on

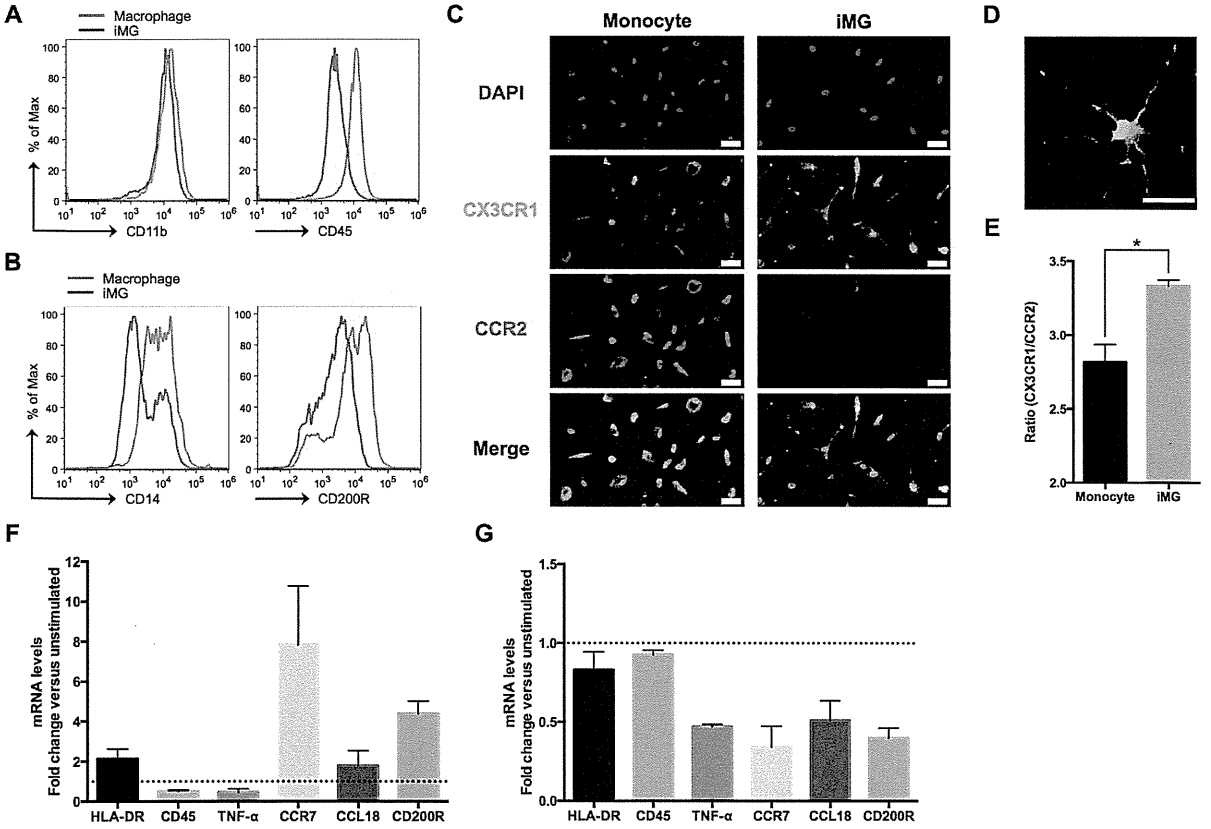

Figure 2 | The iMG cells show the character of human resident microglia. (A and B) The expression levels of surface markers on the iMG cells and induced macrophage were performed by flow cytometer. Peripheral monocytes were incubated with GM-CSF (macrophage) or cocktail of GM-CSF and IL-34 (iMG cells) for 14 days. The iMG cells showed the specific phenotypes of microglia compared to macrophage. (C to E) The expression pattern of CCR2 and CX3CR1 between monocytes and iMG cells were observed by immunocytochemistry. The monocytes and iMG cells were cultured for 14 days, and stained with specific antibodies. (C and D) The iMG cells were stained with bright green fluorescence (CX3CR1) bearing highly branched forms. Scale bar, 50 μm. (E) The expression ratio (CX3CR1/CCR2) of iMG cells was significantly higher than that of monocytes by flow cytometry (n = 3). The iMG cells were incubated with IL-4 (F) or dexamethasone (G) for 72 hours, and extracted RNA was analyzed by qRT-PCR (n = 6). Fold changes were depicted in mRNA levels after stimulation compared with unstimulated cells. $*P < 0.05$. Error bars, standard error of the mean (SEM).

the iMG cells during phagocytosis are significantly higher compared to those on non-treated cells (Fig. 3, B and C).

Analysis of the iMG cells from a patient of NHD. The above results demonstrated that the iMG cells have the dynamic functions of human microglia, and we suggest that iMG cells have the possibility to be utilized for analyzing the underlying microglial pathophysiology of brain disorders. As the initial step, we conducted the first translational analysis of the iMG cells derived from a patient of NHD. NHD is believed to be caused by microglial dysfunction, while no investigation exists using living human microglial cells from patients of NHD. We analyzed the dynamic functions of microglia using the iMG cells from a patient of NHD (141delG in DAP12 gene), after obtaining informed consent (under the permission of the Institutional Review Board of Kyushu University and Osaka University). In agreement with genetic diagnosis, the iMG cells from the NHD patient showed significantly lower expression of DAP12 than those from a healthy control, and there was no difference in TREM2 expression (Fig. 4A). Interestingly, the production of pro-inflammatory cytokines (TNF-α and IL-6) was delayed in the iMG cells from the NHD patient as compared to those from the healthy control after 24 hours. Furthermore, the iMG cells from the patient showed a significantly lower level of anti-inflammatory cytokine (IL-10) than those from the healthy control. The production levels of pro-inflammatory cytokines (TNF-α, IL-6, IL-1β and IL-8) had no significant differences between the NHD and healthy control after 72 hours (Fig. 4B). Next, we examined whether it is possible for iMG cells from the healthy control to silence the target gene with siRNA (DAP12). DAP12 was successfully downregulated in the iMG cells from the healthy control (Supplementary Fig. S2). We assessed the cytokine production of iMG cells treated with siRNA. Predictably, the production of pro-inflammatory cytokines (TNF-α and IL-6) was delayed in the DAP12-silenced iMG cells as compared to the control (Fig. 4C).

Discussion

We have shown a novel technique for developing directly induced microglia-like cells from human peripheral blood cells. GM-CSF and IL-34 converted human monocytes into iMG cells within 14 days. The iMG cells have microglial characterizations; expressing markers, forming a ramified morphology, and phagocytic activity with various cytokine releases. Until now, some attempts to induce microglia-like cells from hematopoietic cells have been performed using an astrocyte co-culture system or astrocyte-conditioned media[29,30], and GM-CSF and IL-34 are known to be derived from astrocytes[30,31].

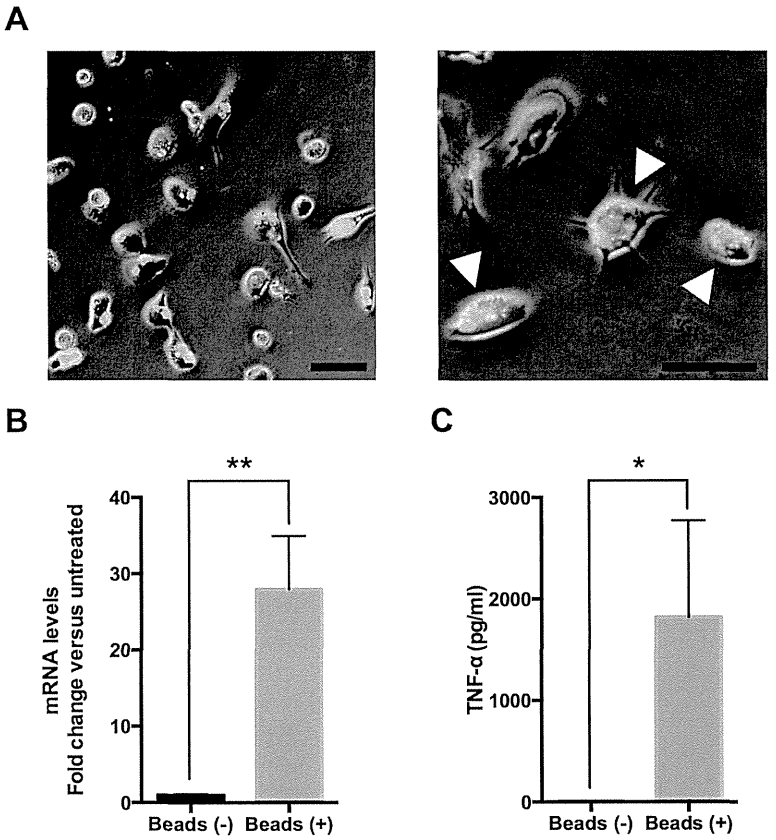

Figure 3 | Dynamic functional analysis of the iMG cells. (A) The iMG cells were incubated with FITC-conjugated latex beads for 24 hours, and phagocytic activity was observed by fluorescent microscopy. The iMG cells showed the ability of phagocytosis with morphological changes into an ameboid form (arrow head). Scale bar, 50 μm. (B and C) The ability of TNF-α production during phagocytosis was measured on the iMG cells. The iMG cells were incubated with latex beads for 72 hours. The extracted RNA and culture supernatant were analyzed by qRT-PCR and Cytometric Beads Array System (CBA), respectively. The mRNA expression (B) and protein level of TNF-α (C) on the iMG cells were significantly higher compared to controls (B, n = 4; C, n = 6). $*P < 0.05$, $**P < 0.01$. Error bars, SEM.

Especially, IL-34 has recently come to be known as a key molecule for the proliferation of microglia[32]. The present data have suggested that both GM-CSF and IL-34 from astrocytes are the minimum essential inducing-factors for microglia-like cells from hematopoietic cells.

The present results indicate that the iMG cells from a patient of NHD show slower (24 h) but not weaker (72 h) pro-inflammatory cytokines' responses compared to those from the healthy control, possibly due to the deletion of DAP12. In addition, suppression of IL-10 production from the iMG cells from the NHD patient indicates that human brain microglia of NHD patients tend to be shifted to pro-inflammatory reactions compared to those of healthy controls. Furthermore, the situation observed in the iMG cells from a NHD patient was replicated with iMG cells from a healthy control using siRNA. These data have suggested that DAP12 expression is a key factor in from the perspective of microglial immunoresponse such as cytokine production in NHD patients. In the present study, we examined the iMG cells from a female patient of NHD. Recent studies have suggested microglial functional differences between sexes[33]. Further investigations should focus on sex differences related to microglial dysfunction of NHD. DAP12 and TREM2 are the responsible genes of NHD, which mediate various important roles such as phagocytosis and cytokine production in osteoclasts, macrophages, dendritic cells and microglia[34]. A rodent study showed that deletion of DAP12 induces synaptic impairments due to microglial dysfunction[15]. Hamerman et al.[35] demonstrated that macrophage from DAP12-deficient mice increase inflammatory cytokines' responses, which suggest that DAP12-deleted microglia increase similar inflammatory response. These previous reports and our present findings based on the iMG cells from a NHD patient suggest that human NHD microglia has the potential to induce stronger and long-acting pro-inflammatory reactions compared to those of healthy human subjects.

In sum, we have shown a novel technique of developing directly induced microglia-like cells, named "iMG cells", with a combination of GM-CSF and IL-34 from adult human monocytes, easily and quickly without any virus, feeder cells, and genetic engineering. The iMG cells proved to have many characterizations of microglial cells, such as expressing $CD11b^{high}/CD45^{low}$ and $CX3CR1^{high}/CCR2^{low}$. Moreover, the iMG cells expressed dynamic functions such as phagocytosis and releasing pro- and anti- inflammatory cytokines. Further investigations such as microarray analysis should be conducted to validate the closeness of iMG cells to human primary microglial cells in the brain. Finally, we presented the translational utilities of the iMG cells for analyzing the underlying microglial pathophysiology of NHD. We believe that this novel technique will shed new light on solving unknown dynamic aspects of human microglial cells in various brain disorders.

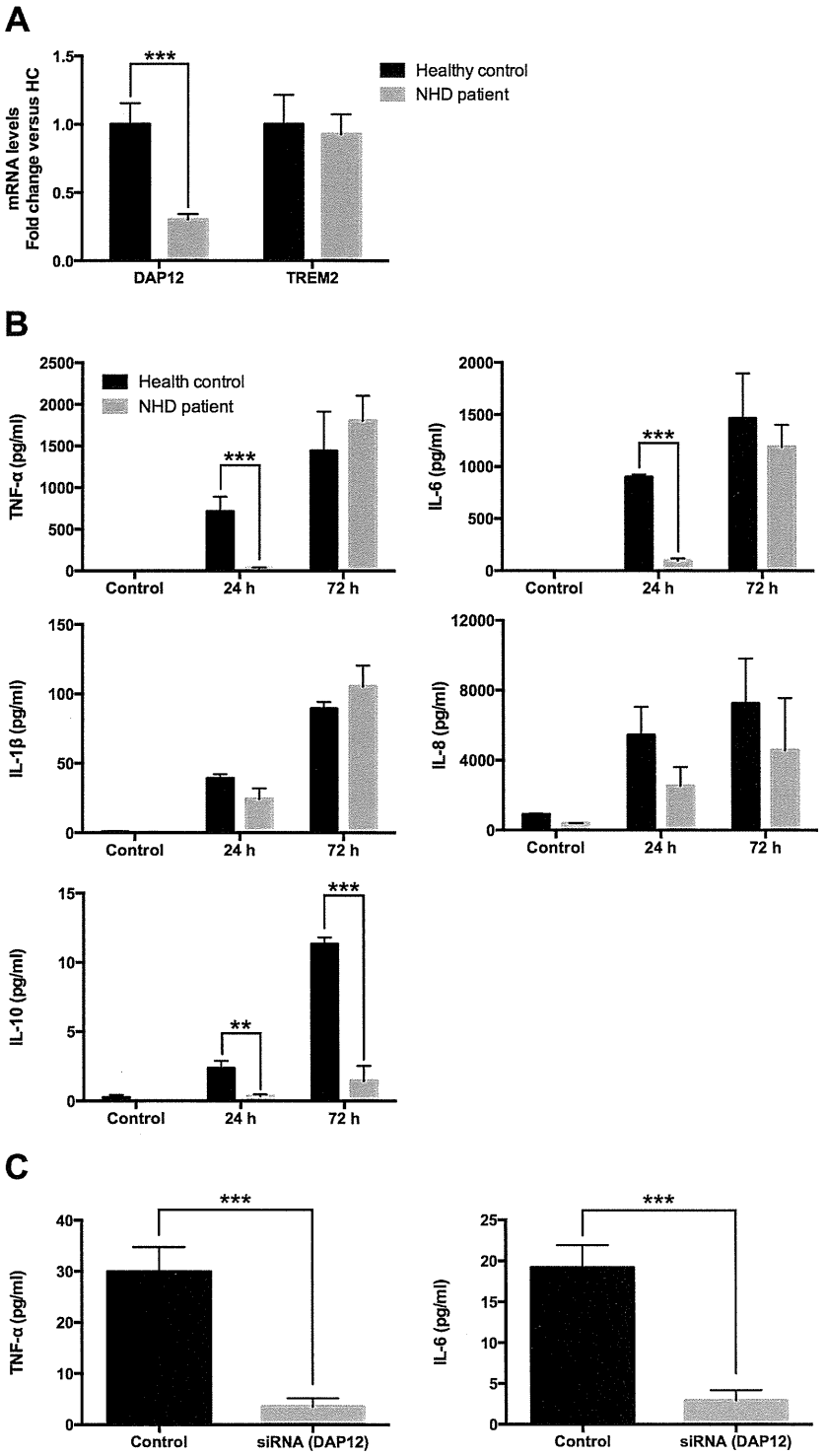

Figure 4 | Dynamic functional analysis of the iMG cells from a patient of NHD. (A) The iMG cells from the NHD patient showed significantly lower gene expression of DAP12 compared to those from the healthy control (n = 6). (B) Cytokine production from the iMG cells were compared between the NHD patient and the healthy control. The iMG cells from the NHD patient and the healthy control were incubated with latex beads for 24 or 72 hours, and culture supernatants were analyzed by CBA. In the iMG cells from the NHD patient, the production of pro-inflammatory cytokines (TNF-α and IL-6) was delayed, and that of anti-inflammatory cytokine (IL-10) was decreased (n = 4). (C) The effects of DAP12 silencing on the proinflammatory cytokine production. The iMG cells treated with siRNA were incubated with latex beads for 24 hours, and culture supernatants were analyzed by CBA In the iMG cells treated with siRNA, the production of pro-inflammatory cytokines was delayed (n = 8) $^{**}P < 0.01$, $^{***}P < 0.001$. Error bars, SEM.

Methods

Subjects. The present study was conducted in accordance with the World Medical Association's Declaration of Helsinki and was approved by the Ethics Committee of the Graduate School of Medical Sciences, Kyushu University and Osaka University. We recruited a middle-aged female patient, who was diagnosed with Nasu-Hakola disease (141delG in DAP12 gene) in her thirties. Based on informed consents both from the patient and a family member, we took a blood sample. Healthy adult volunteers including an age-matched female were also recruited.

Induction of induced microglia-like (iMG) cells from human peripheral blood. Peripheral blood was collected using a heparinized tube from healthy adult volunteers and a patient of NHD. Peripheral blood mononuclear cells (PBMC) were isolated by Histopaque-1077 (Sigma Chemical Co., St. Louis, MO) density gradient centrifugation. PBMC were resuspended with RPMI-1640 (Nacalai Tesque, Kyoto, Japan), 10% heat-inactivated fetal bovine serum (FBS; Endotoxin = 0.692 EU/ml; Japan Bio Serum, Hiroshima, Japan) and 1% antibiotics/antimycotic (Invitrogen, Carlsbad, CA). PBMC were plated onto culture chambers at a density of 4×10^5 cells/ml and cultured overnight in standard culture conditions (37°C, 5% CO_2). After overnight incubation, culture supernatant and non-adherent cells were removed. The adherent cells (monocytes) were cultured with RPMI-1640 Glutamax (Invitrogen) supplemented with 1% antibiotics/antimycotic and a mixture of the following candidate cytokines; recombinant human GM-CSF (10 ng/ml; R&D Systems, Minneapolis, MN), recombinant human IL-34 (100 ng/ml; R&D Systems) and M-CSF (10 ng/ml; Peprotec, Rocky Hill, NJ) in order to develop iMG cells. We also developed induced macrophage from human monocytes; monocytes were cultured with RPMI-1640 Glutamax supplemented with 1% antibiotics/antimycotic and recombinant human GM-CSF (10 ng/ml). All cells were cultured in standard culture conditions for up to 14 days.

Cell morphology. Morphological changes of cytokines-treated cells were examined using phase-contrast microscopy (TS100-F; Nikon Instech, Tokyo, Japan). Images were taken with a DS-Vi1 digital camera (Nikon Instech) and a DS-L3 control unit (Nikon Instech).

Flow cytometry. Flow cytometry was performed using a FACS Aria (BD Biosciences, Bedford, MA) with FACS Diva software (BD Biosciences). Flow cytometry data were analyzed using FlowJo software (Tree Star, San Carios, CA). For iMG phenotyping, fluorochrome conjugated monoclonal antibodies specific for human CD11b (APC-Vio770; Miltenyi Biotec, Gladbach, Germany), CD14 (FITC; Sigma), CD45 (PE; Miltenyi Biotec) and CD200R (Alexa647; Serotec, Oxford, UK) were used. Induced macrophage and iMG cells were cultured in 6-well plates (Corning, NY) at a density of 4×10^5 cells/ml. Cells were harvested by non-enzymatic cell dissociation solution (Sigma) and cell lifter (Corning). The cells were washed with MACS buffer (Miltenyi Biotec) and incubated for 5 minutes at 4°C in FcR-blocking reagent (Miltenyi Biotec). Antibodies were incubated with cell suspension for 30 minutes at 4°C, washed with calcium-magnesium-free phosphate-buffered saline (PBS(-)), resuspended and fixed with 1% paraformaldehyde (Wako, Osaka, Japan) in PBS(-). The fluorescence intensity of the cells was measured.

Indirect immunofluorescence for flow cytometry was performed using the following antibodies: rabbit anti-CX3CR1 antibody (Immuno-Biological Laboratories, Gunma, Japan) and mouse anti-CCR2 antibody (R&D Systems). The monocytes and iMG cells were treated with the same process until the primary antibody staining. After primary staining, washed with MACS buffer and were stained with Alexa488- or Alexa546-conjugated secondary antibodies (Invitrogen). The ratio of CX3CR1 to CCR2 was calculated by the fluorescent intensity of each fluorochrome.

Immunocytochemistry. In immunocytochemistry, iMG cells and monocytes were cultured in 8-well chambers (LabTec chamber slide system; Nalge Nunc International, Rochester, NY) at a density of 4×10^5 cells/ml. These cells were fixed with 4% paraformaldehyde (Wako) for 20 minutes and then rinsed thrice with PBS(-) for 5 minutes. Indirect immunofluorescence was performed using the following antibodies: rabbit anti-CX3CR1 antibody (1 : 500 dilution; Immuno-Biological Laboratories, Gunma, Japan) and mouse anti-CCR2 antibody (1 : 500 dilution; R&D Systems). Cells were incubated in primary antibodies diluted in 0.1% Triton-X 100 in PBS containing 5% normal goat serum at 4°C overnight. After rinsing thrice with PBS(-) for 5 min, Alexa488- or Alexa546-conjugated secondary antibodies (Invitrogen) were used for detection. Fluorescent images were taken with a confocal laser scanning microscope (LSM-780; Carl Zeiss, Jena, Germany).

Quantitative real time-polymerase chain reaction (qRT-PCR). To assess the gene expression patterns in iMG cells after the treatment of IL-4, dexamethasone or during phagocytosis, we performed qRT-PCR using a LightCycler 480 system (Roche Diagnostics, Mannheim, Germany). IL-4 (40 ng/ml; Peprotec), dexamethasone (2 nM; Sigma) or latex beads-rabbit IgG-FITC solution (Cayman Chemical) was added to the iMG cells and incubated for 72 hours in standard culture conditions. After incubation, the iMG cells were washed and the total RNA was extracted using a High Pure RNA Isolation kit (Roche Diagnostics) according to the manufacturer's protocol, and subjected to cDNA synthesis using a Transcriptor First Strand cDNA Synthesis kit (Roche Diagnostics). qRT-PCR for HLA-DR, CD45, TNF-α, CCR7, CCL18 and CD200R was performed using each primer (Supplementary Table 1). Beta 2-microglobulin of Universal ProbeLibrary (Roche Diagnostics) was used as a house-keeping control gene. Fold changes were depicted in mRNA levels after stimulation compared with unstimulated cells.

Using the iMG cells from a female NHD patient and an age-sex matched healthy control, we examined the gene expression of DAP12 and TREM2 by qRT-PCR. The iMG cells were washed and the total RNA was extracted respectively, and qRT-PCR was performed using each primer (Supplementary Table 1). Beta 2-microglobulin was used as a house-keeping control gene. Fold changes were depicted in mRNA levels after stimulation compared with unstimulated cells.

Phagocytosis. Phagocytosis was examined by fluorescent microscopy using Phagocytosis Assay Kit (Cayman Chemical, Ann Arbor, MI) according to the manufacturer's protocol. The iMG cells were cultured in 8-well chambers (Nalge Nunc International) at a density of 4×10^5 cells/ml. We added 50 μl of the latex beads-rabbit IgG-FITC solution to each well of the chamber, and incubated the cells in standard culture conditions for 24 hours. After discarding the supernatant by careful aspiration, we quenched surface-bound fluorescence, added 125 μl of trypan blue solution to each well of the chamber, and incubated for two minutes at room temperature. Each well was analyzed by using a fluorescence microscope (Olympus IX-71, Tokyo, Japan) and DP71 digital camera system (Olympus).

Cytokine measurement. Secretion of pro- and anti- inflammatory cytokines (TNF-α, IL-1β, IL-6, IL-8 and IL-10) during phagocytosis was measured from culture supernatants using Cytometric Beads Array System (CBA; BD Biosciences) according to the manufacturer's protocol. Latex beads-rabbit IgG-FITC solution (Cayman Chemical) was added to the iMG cells and incubated for 24 or 72 hours in standard culture conditions. After incubation, culture supernatants were centrifuged at 10000 × g for 10 minutes and kept frozen at −80°C until assayed. The culture supernatants were incubated with the cytokine capture beads and PE-conjugated detection antibodies for 3 hours at room temperature. Afterwards, the capture beads were washed and measurement data were acquired using a FACS Canto™ flow cytometer (BD Biosciences). The data analysis was performed using FCAP Array software (BD Biosciences).

Gene silencing of DAP12. Gene silencing assay was performed using siRNA (DAP12; Santa Cruz, USA) and RNAiMAX (Invitrogen) according to the manufacturer's protocol. The mix solution of siRNA and RNAiMAX was added to the iMG cells. After overnight incubation, the medium was changed to the culture medium and incubated for 48 hours. The siRNA-modified iMG cells were used for cytokine assay.

Statistical analysis. Analysis of comparisons between groups were conducted by two-tailed Student's t-test.

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Author contributions

All authors contributed substantially to the scientific process leading up to the writing of the present paper. T.A.K., the principal investigator of the present research, and M.O., the first author, created the conception and design of the project and wrote the protocol. The performance of experiments and the data analysis/interpretation were performed by M.O., T.A.K., D.S., N.S., K.H. and N.S. M.O. wrote the first draft of the manuscript. Clinical recruitments were conducted by R.H., K.S., T.Y., K.H. and N.S. Critical revisions of the manuscript were made by T.A.K., D.M., H.U. and S.K. All authors have approved to submit the final manuscript.

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Neuron-glia interaction as a possible glue to translate the mind-brain gap: a novel multi-dimensional approach toward psychology and psychiatry

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Neurons and synapses have long been the dominant focus of neuroscience, thus the pathophysiology of psychiatric disorders has come to be understood within the neuronal doctrine. However, the majority of cells in the brain are not neurons but glial cells including astrocytes, oligodendrocytes, and microglia. Traditionally, neuroscientists regarded glial functions as simply providing physical support and maintenance for neurons. Thus, in this limited role glia had been long ignored. Recently, glial functions have been gradually investigated, and increasing evidence has suggested that glial cells perform important roles in various brain functions. Digging up the glial functions and further understanding of these crucial cells, and the interaction between neurons and glia may shed new light on clarifying many unknown aspects including the mind-brain gap, and conscious-unconscious relationships. We briefly review the current situation of glial research in the field, and propose a novel translational research with a multi-dimensional model, combining various experimental approaches such as animal studies, *in vitro* & *in vivo* neuron-glia studies, a variety of human brain imaging investigations, and psychometric assessments.

Keywords: translational research, neuron-glia interaction, mind-brain gap, unconscious, neuropsychoanalysis

INTRODUCTION

Neurons and synapses have long been the dominant focus of neuroscience, thus the pathophysiology of psychiatric disorders has come to be understood within the neuronal doctrine. However, the majority of cells in the brain are not neurons but glial cells including astrocytes, oligodendrocytes, and microglia. Traditionally, neuroscientists regarded glial functions as simply providing physical support and maintenance for neurons. Thus, in this limited role glia had been long ignored (1). Recently, glial functions have been gradually investigated, and increasing evidence has suggested that glial cells perform important roles in various brain functions. Digging up the glial functions and further understanding of these crucial cells, and the interaction between neurons and glia may shed new light on clarifying many unknown aspects including the mind-brain gap, and conscious-unconscious relationships. In addition, glial pathophysiology may explain the possible implications for the pathogenesis of major psychiatric disorders. The complexity of these aspects has yet to be well investigated. To explore these physiological and pathological aspects, novel translational methods should be applied with a multi-dimensional approach. Herein, we will briefly review the current situation of glial research in the field, and propose a novel translational research with a multi-dimensional model, combining various experimental approaches such as animal studies, *in vitro* & *in vivo* neuron-glia studies, a variety of human brain imaging investigations, and psychological/psychiatric assessments.

GLIAL ROLES AND PATHOLOGY IN PSYCHIATRIC DISORDERS

Recent biological studies have been revealing the important roles of glial cells in the process of neuropsychiatric disorders.

ASTROCYTES

Astrocytes are the most prevalent cell type in human brain and contribute to the homeostasis of the brain by regulation of neuronal metabolism, modulation of CNS inflammation, and direct/indirect synaptic transmission such as MNDA receptors (2, 3). Astrocyte dysfunction has been critical for various neurological disorders (4). Recent studies have shown abnormal expression of glial fibrillary acid protein (GFAP) – a prototypical marker of astrocyte – in postmortem brain of patients with schizophrenia and major affective disorders (5–7). In addition, recent rodent studies have suggested that astrocytes modulate anxious and depressive behaviors (8, 9). On the other hand, direct modulating effects of antidepressants have also been revealed (10–13). Thus, astrocytes have been supposed to be a novel therapeutic target against various psychiatric disorders such as major affective disorders and bipolar disorders (14, 15).

OLIGODENDROCYTES

Oligodendrocytes contribute to brain development and homeostasis in the brain by formulating myelin around axons, supporting neuronal networks in the brain. Recently, novel oligodendrocyte functions have been revealed such as monitoring neuronal

activities via myelin-forming oligodendrocytes (16) and modulating the conduction velocity of action potentials along axons in the rat hippocampus (17). Dysfunctions of oligodendrocytes have been indicated in psychiatric disorders, especially schizophrenia and major affective disorders, from a series of genetic studies (18, 19), postmortem studies (20–22), and diffusion tensor imaging (DTI) studies (23–27). A novel animal model of schizophrenia has been developed by treating a copper chelator, which induces oligodendrocyte dysfunction and white matter abnormality as demyelination and schizophrenia-related behaviors (28, 29). Cuprizone caused marked behavioral changes (working memory deficit) indicated by the results of Y-maze task, which showed an increase in the number of arm entries and a decrease in alternation behavior. These cuprizone-induced behavioral changes were effectively prevented by chronic administration of quetiapine, an atypical antipsychotic, which also diminished demyelination (28). On the other hand, recent rodent studies have revealed the interaction between oligodendrocyte dysfunction and social behaviors. Makinodan et al. reported that oligodendrocyte dysfunction is formed by early-period social isolation and this maladaptive environment induces working memory deficit associated with prefrontal cortex (PFC) function in later life (30). Liu et al. reported that protracted social isolation of adult mice induces behavioral, transcriptional, and ultrastructural changes in oligodendrocytes of the PFC and impairs adult myelination (31).

MICROGLIA

Microglia are unique glial cells of mesodermal origin in the brain that act as "brain macrophage"; immunological/inflammatory players by moving around and releasing cytokines and free radicals (32, 33). Thus, microglia have proved to play important roles in various brain pathologies such as neurodegenerative diseases and neuropathic pain via inducing inflammation and oxidative stress (34–36). Recently, microglia have been revealed to have direct contact with synapses and have proved to play crucial roles in neuronal development through synaptic pruning (37–39). Postmortem studies have shown microglial activation in the brain of patients with schizophrenia and major affective disorders, especially suicide victims (40–42). In addition, positron emission tomography (PET) imaging studies using the peripheral benzodiazepine receptor bindings has shown that microglia are activated in patients with schizophrenia (43–45) and autism (46). On the other hand, minocycline, an antibiotic with inhibitory effects on microglial cells, has been reported to have therapeutic effects on schizophrenia and unipolar psychotic depression (47–49). In addition, rodent *in vitro* studies have proved the novel effect of psychotropic drugs (atypical antipsychotics such as risperidone and aripiprazole, and antidepressants such as paroxetine and sertraline, both selective serotonin reuptake inhibitors) directly on microglia by suppressing release of inflammatory cytokines and free radicals (50–54). Thus, microglia are suggested to play key roles in psychiatric disorders (53, 55, 56).

In the brain, neurons, astrocytes, oligodendrocytes, and microglia are mutually communicating with each other, by direct-contacting or via neurotransmitters and other various small molecules (57), and dysfunction of neuron-glia communication may induce pathological conditions not only in neurodegenerative diseases (58) but also in psychiatric conditions such as psychosis, depression, and anxiety. The above-mentioned recent findings strongly suggest that glial cells contribute to psychiatric disorders, while the underlying mechanisms have not been clarified.

POSSIBLE GLIAL ROLES IN HUMAN MENTAL FUNCTIONS

Until recently, the actual roles of glia in mental activities, especially for healthy humans, have not been investigated. As the first step to clarify this unexplored field, we have started to conduct a series of social decision-making experiments with healthy human subjects using minocycline, a microglial inhibitor (59–61). Healthy Japanese adult males made a monetary decision about whether or not to trust an anonymous partner after a 4-day oral administration of minocycline. Our first trial revealed that the minocycline group showed a positive correlation between their monetary score in trust game and their evaluation scores of others' trustworthiness in a questionnaire (Yamagishi's General Trust Scale), but surprisingly the placebo group did not (60). It would be rational to consider the monetary and questionnaire score to be positively correlated because both scores measure the other's trustworthiness, but there was no positive correlation with the placebo group. The questionnaire is measuring only conscious-level decision-making, on the other hand the monetary score is measuring the final decision-making affected by not only the conscious but also the unconscious; suggesting that some unconscious noisy factors seem to be affecting the placebo group. Treatment with minocycline, a microglial inhibitor, has shown the positive correlation. Therefore, this first trial has indicated that microglial activation may cause "unconscious noises" against appropriate social decision-making, and inhibiting microglial activity may reduce such noise (60). In a next trial with larger samples, we additionally measured the effects of anxiety and personality as candidates for "noise" factors, by using Temperament and Character Inventory (TCI) and State-Trait Anxiety Inventory (STAI) (59). The monetary score in trust game was significantly lower in the minocycline group. Interestingly, participants' ways of decision-making were significantly shifted; certain personality traits (cooperativeness, reward dependence, and self-directedness) proved to be the main modulating factors of decision-making in the placebo group, on the other hand the minocycline group was mainly modulated by state anxiety and trustworthiness. Our results of the second trial suggest that minocycline led to more situation-oriented decision-making, possibly by suppressing the effects of personality traits, and furthermore that personality and social behaviors might be modulated by microglia. Interestingly, cooperativeness has proved to be the most influential factor in the process of decision-making in the placebo group of Japanese participants (59). It is widely known that cooperativeness and cooperative behaviors have been highly respected and emphasized aspects in Japanese society. Thus, of course, these aspects are ingrained during childhood by various sociocultural experiences within family relationships, schools, and other areas of society in Japan. Early-life events may activate human microglia, establish a certain neurosynaptic connection, and this formation may determine personality and personality-oriented social behaviors in later life (59, 62). If these experiments are conducted in other countries with different sociocultural backgrounds, other personality traits may be identified.

In addition, we have recently reported a possible outcome that minocycline, a microglial inhibitor, also reduces the risk of the "honey trap" during economic exchanges between males × females (61). Males tend to cooperate with physically attractive females without careful evaluation of their trustworthiness. In our experiment, young healthy male participants made risky choices (whether or not to trust female partners, identified only by photograph, who had decided in advance to exploit the male participants). The results show that trusting behavior in male participants significantly increased in relation to the perceived attractiveness of the female partner, but attractiveness did not impact trusting behavior in the minocycline group (61). These novel effects of minocycline may highlight the unknown roles microglia play in deeper human mental activities; microglia may modulate our unconscious drives in various social settings. The above-mentioned findings shed new light on the dark side of microglial social/mental functions in humans, especially highlighting the role of microglia for the unconscious. In the same way that Sigmund Freud, the founder of psychoanalysis, proposed that our behaviors must be controlled by the unconscious world, microglia may unconsciously control our behaviors. How do microglia act as fundamental mediators between the conscious and the unconscious world? What do neurobiological mechanisms justify their eventual role in bridging the gap between neuroscience and psychoanalysis? Answers to the above questions are not yet clear, but we have recently proposed a hypothesis creating a link between Freud's unconscious drives such as the death drive and microglial activation (62). For example, microglial maladaptive over-activation in a certain brain region may activate human aggressive behaviors as a result of destructive drives [For the details, please see our recent article; Ref. (62)]. In the brain, not only microglia but also other glia such as astrocytes and oligodendrocytes exist, thus complicated neuron-glia interactions may modulate our mental activities including the unconscious (**Figure 1**). Further research should be applied to clarify these unresolved questions.

After Freud's theory of unconscious roles in behaviors which was initially identified in the 1980s (63), Pribram and his colleagues have developed this theory in terms of a better articulated model of neural computation (64, 65). In addition, recent neuropsychoanalytic movements have been updating Freud's theory with modern sophisticated methods of cognitive neuroscience (66–72). Thus, these recent approaches have been revealing the underlying mechanisms of implicit processing in a variety of information-processes including the social processes using rodent experiments. At present, the link underlying mechanisms between neuron-glia interactions and the conscious-unconscious relationship is largely unsolved, and few experimental methods have been developed to test these unknown brain mechanisms at either the microscopic or macroscopic level. Unconscious processing needs to be given a greater focus in terms of brain mechanisms. One possible solution is the novel ontogenetic approach; called "optogenetics" (73–76). Optogenetics is a revolutionary technique involving taking a light-activated gene (called a channel rhodopsin) targeted into a single neuron type. This technique enables to clarify direct interaction between activation of specific neuron in specific region by light and the resulting outcomes such as behaviors and emotional reactions at rodent level. A recent study has interestingly

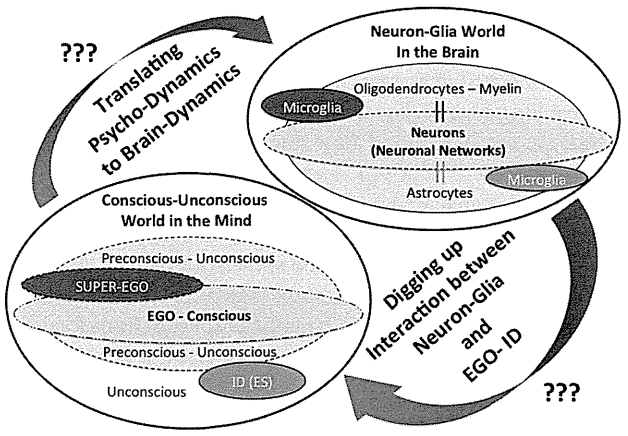

FIGURE 1 | The mind-brain gap from a novel glial neuropsychoanalytic perspective. The interaction between the mind and the brain has not been well understood. Freud, the founder of psychoanalysis, proposed the conception of mind structure models consisting of the following three components: *the ID* (unconscious/instinctual drives), *the EGO* (the exclusive apparatus of the conscious mind), and *the SUPER-EGO* (which represses *the id* in order to avoid any disruptions of rational thought). The existence and the significances of these mental components may be explained by future understandings of the neuron-glia interactions. The hypothetical interaction between the ID (unconscious drives) and microglia has already been proposed in our recent theoretical paper (62).

shown that activation of specific neurons in hippocampus produce a false memory in mice (77). Further technological developments in modulating glial cells by light and in activating both neurons and glial cells at the same time, by multiple fluorescent lights, may shed new light on resolving unknown roles of glia and neuron-glia interaction in behaviors and the conscious-unconscious. Functional roles and pathological contributions of astrocyte, oligodendrocyte, and/or microglia in conscious or unconscious processes have not been well understood, and we hypothesize that each cell may differently contribute to these physical and/or pathological processes in different brain regions such at the brainstem, limbic, or thalamocortical region, respectively. Future developments in optogenetics may clarify these unknown aspects.

LIMITATION AND FUTURE PERSPECTIVES OF NEURO-GLIA RESEARCH ON PSYCHOLOGY AND PSYCHIATRY

To explore the above-mentioned hypothesis, further translational research is needed. Several limitations should be made note of at the present stage. At first, rodent studies focusing on the unconscious are limiting. Even if the unconscious exists in rodents, it seems to be impossible to measure the unconscious in rodents devoid of human language capabilities. Therefore, to uncover the unconscious mechanisms, we have no alternative method except examining actual human subjects. We have no specific drugs to modulate glial cells utilized in human, and minocycline is reported to have other brain functions in addition to microglial inhibition (78, 79). On the other hand, some brain imaging techniques enable us to explore the unknown roles of glial cells such as DTI technique and PET imaging using the peripheral benzodiazepine receptor bindings, while the specificities of these imaging methods are not

at satisfactory levels (80). On the other hand, we can reconsider previous findings of brain imaging experiments. Functional MRI (fMRI) is a brain imaging procedure measuring brain activity by detecting associated changes in blood flow (81, 82). Outcomes of fMRI have long been believed to monitor solely neuronal activities, because cerebral blood flow and neuronal activation have been thought to be almost equivalent. However, not only neuronal activities but also glial activities, especially astrocyte activities, rely on cerebral blood flow. Therefore, at least to some extent, brain activities expressed by fMRI may be showing a part of glial activation. In addition, MR spectroscopy (MRS) is one of the novel imaging approaches to measure dynamic brain functions focusing on metabolomics including glia-related molecules. For example, myo-inositol, which can be measured by MRS, is regarded as a marker of astrocyte activity (83). These imaging methods and combination of these imaging techniques may shed new light on clarifying unknown roles of glia in psychiatric disorders (84, 85). For example, activated microglia-derived myelin damage has been indicated in the pathophysiology of schizophrenia by rodent experimental models (28, 29, 86, 87), while it is not confirmed in human subjects. Combination of human DTI and PET may clarify the mutual interaction between microglial activation and myelin damage in schizophrenia patients. On the other hand, connectivity of each brain region has been important in the understanding of the roles of brain functions from the era of Hughlings Jackson. fMRI studies have revealed the importance of these aspects (88, 89), and the recent development of DTI is showing us the significance of more complicated brain networks focusing on not only neurons but also glial cells such as oligodendrocytes (90, 91).

Finally, we propose the multi-dimensional approach to clarify the underlying brain mechanisms of mental functions including the unconscious (**Figure 2**). Based on our discussion, we believe that not only neurons but also glial cells have a vital role in the process of mental activities, a novel approach focusing on neuron-glia interactions should be applied. Combination of brain imaging techniques focusing on both neurons and glial cells should be applied (24, 26, 27, 43–46, 92–94). The most significant limitation in human brain research is that we cannot obtain living brain cells, including glial cells, from living human subjects from an ethical perspective. Presently, we can apply an alternative method; human brain cells such as neuronal cells can be established from somatic cells (not from the brain) such as skin fibroblasts by utilizing the gene-modification technique of induced pluripotent stem (iPS) cells. In addition, recently, neuronal cells are more easily established from directly conversion of human skin fibroblasts, called induced neuronal (iN) cells (95–99). Novel methods of establishing glial cells are strongly warranted based on iPS or direct conversion techniques in the near future. Multi-dimensional aspects of same human subjects, from genes, blood, brain imaging, psychometrics, social function, unconscious functions, psychodynamic assessments to molecular functions of somatic tissue-derived neuronal and glial cells, should be investigated and analyzed together (**Figure 2**). This approach may explore the novel roles of glial cells in various human mental activities including the unconscious. The application of this method for psychiatric patients should also be encouraged in the establishment of novel diagnostic methods and novel therapies.

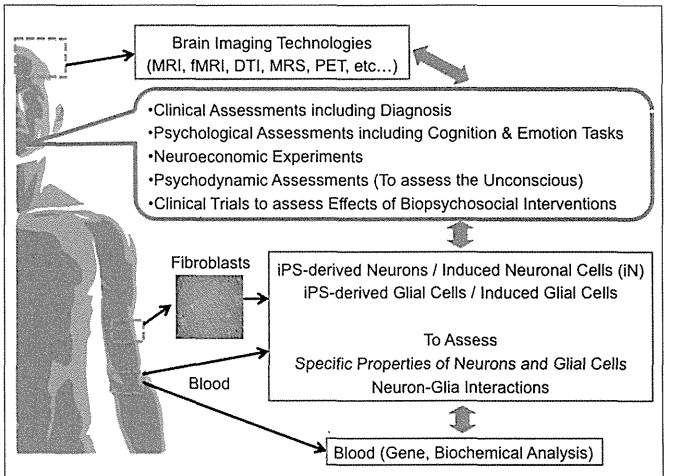

FIGURE 2 | A novel multi-dimensional approach toward psychology & psychiatry.

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