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Innate immune restoration after major surgery in the elderly: functional adaptation for maintaining immune reactivity

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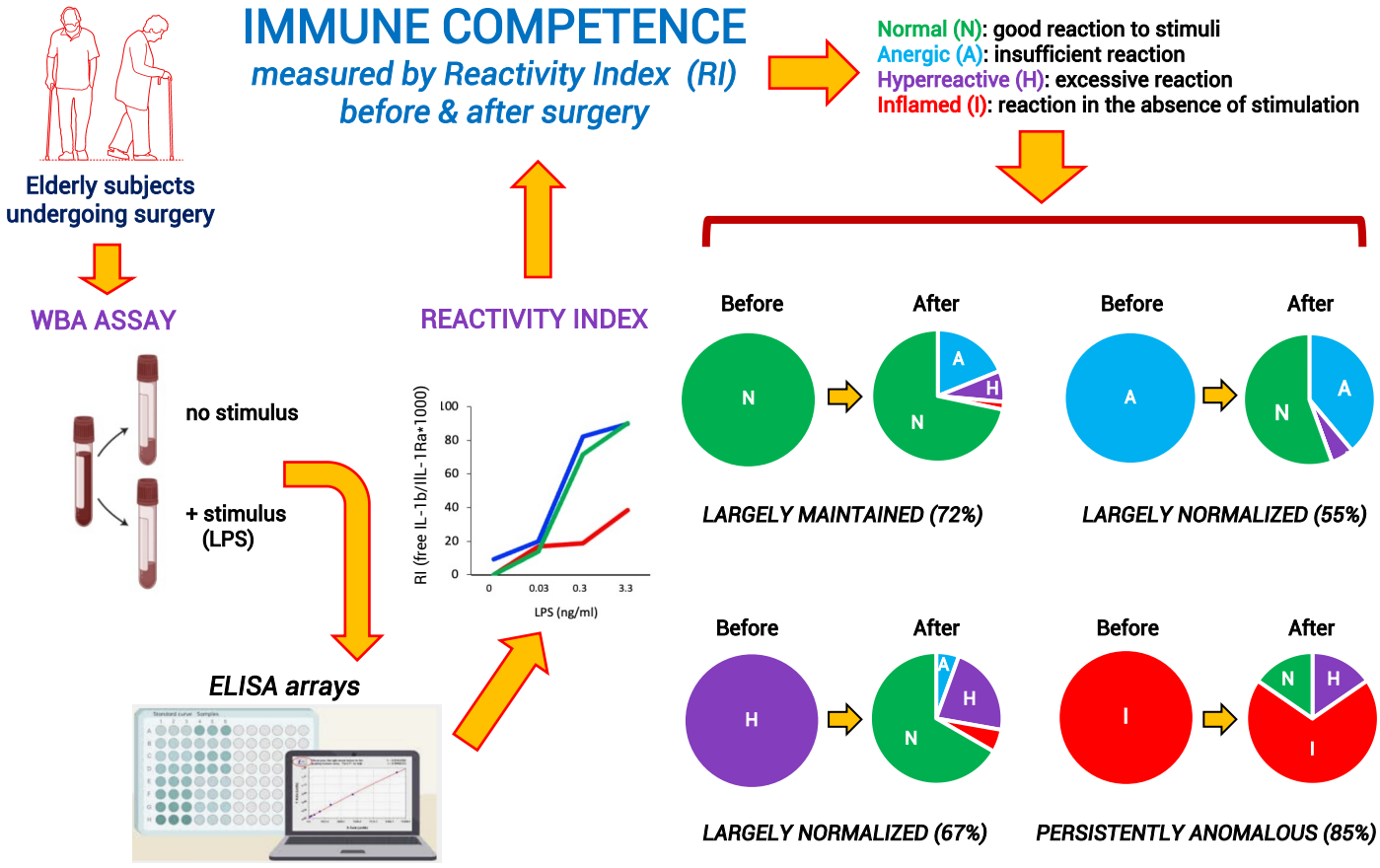
GRAPHICAL ABSTRACT



PUBLIC SUMMARY

- A new reactivity index allows to group elderly subjects into immunologically competent vs. anomalous.
- After major physical stress (surgery), over 50% of initially anomalous subjects normalize.
- Constitutively inflamed subjects do not normalize.
- Normalization is due to active reprogramming of the production of inflammatory vs. anti-inflammatory factors.
- Stress-induced generation of innate memory is suggested as adaptation mechanism to attain immune competence.

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Innate immune restoration after major surgery in the elderly: functional adaptation for maintaining immune reactivity

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Innate immune memory reflects the capacity of innate immune cells to undergo functional reprogramming after challenges. Given its importance as protective immune mechanism in the elderly, in this study we aimed to characterize the development of protective functional innate memory upon major surgery in elderly patients. In a prospective cohort of 102 elderly patients undergoing major surgery, innate immune reactivity was measured as blood leukocyte response to bacterial LPS one day before surgery and 3 months post-operatively, using free active IL-1β as reactivity index. Based on their response, patients were initially classified into four functional response categories: Normal (n=53), Anergic (n=18), Hyperreactive (n=18), and Inflamed (n=13). Thirty-two circulating biomarkers and 29 clinical parameters were analyzed for association with immune state and trajectory. Three months post-surgery, substantial improvement occurred, with 55% Anergic and 67% Hyperreactive patients reaching immune normalization, although some patients maintained abnormal response and some adopted an abnormal reactivity. Irrespective of trajectory, the post-surgical reactivity index was the result of a re-directioning of the production of inflammatory *vs.* anti-inflammatory factors, suggestive of the establishment of functional innate immune memory. Among clinical and biochemical variables, age emerged as the only significant predictor of immune trajectory in initially Normal patients, with individuals >75 years showing a higher risk of post-operative anergy. This study suggests for the first time that surgery-related stress could induce a functional cell-intrinsic memory-like immune modulation that improves subsequent immune responsiveness in elderly individuals. Thus, surgery-induced innate memory can be considered as an adaptation mechanism for maintaining/attaining optimal immune functionality. The maladaptive post-operative immune reactivity observed in some individuals stresses the need for future personalized interventions to re-establish the capacity of optimal adaptation.

INTRODUCTION

This study aims at exploring the possibility that major surgery could induce a functional innate immune memory in elderly patients, directed to maintaining/improving their immune competence.

Innate immune memory is the immune process that changes the non-specific response of innate immune cells (such as monocytes and macrophages) into a more protective reaction to subsequent challenges. This phenomenon has been widely reported in invertebrates, where it also shows a substantial degree of specificity.[1-7] In mammals, it was reported for the first time by the seminal work of Paul Beeson in 1946[8] and supported by several demonstrations in the following years,[9-17] until its more recent revamping with the new name of "trained immunity".[18] While innate memory aims at affording improved protection upon repeated exposure to dangerous agents,[19] it can also be linked to anomalous pathological reactivity (*e.g.*, autoimmune and inflammatory diseases).[20,21] In human beings, innate immune memory is generally non-specific, *i.e.*, it improves innate/inflammatory reactivity to different types of challenges and it can be induced by several types of stressful events, mostly infectious agents and their components.[22] Innate memory is mainly based on epigenetic changes,[23] thus in principle it can last very long, as epigenetic changes can be transmitted to daughter cells and also to the progeny, but it could also have very short duration, since epigenetic modifications can be erased or replaced by different ones in response to new microenvironmental cues.[7] In this scenario, it is hypothesized that exposure to a variety of different stimuli during life can bias, in a personalized fashion, the individual response to new challenges ("immunobiography").[24] This innate memory bias may be particularly evident in elderly people, possibly contributing to their higher levels of basal inflammation ("inflammaging")[24,25] and conditioning their reactivity to new challenges, both in terms of immediate reaction and in the capacity to modulate their innate memory profile. Activation and regulation of innate immunity in elderly people are of particular importance, since innate immunity is less affected by senescence, although prone to reversible exhaustion, opposite to the substantial senescence of adaptive immune responses.[26-29] As a consequence, we can infer that a large part of immune protection in the elderly is based on the efficacy of innate immunity and innate memory, in particular dependent on myeloid cells (since immune aging significantly correlates with myeloid skewing). Notably, monocytes and macrophages in elderly people are in general less reactive to

inflammatory stimuli and more involved in anti-inflammatory functions, most likely as compensatory mechanism in a scenario of increased tissue inflammation and damage.[26-29]

In this study, we have examined the generation/modulation of functional innate memory in a cohort of 102 elderly patients undergoing surgery, aiming to identify possible memory-dependent long-term effects on innate immunity. Surgery was taken as memory-inducing/modulating event. Memory-like functional effects were assessed as changes in the immune reactivity index, *i.e.*, the capacity of blood leukocytes to respond to an *in vitro* challenge with a bacterial agent (gram-negative lipopolysaccharide, LPS) in terms of production of free active IL-1β, calculated as the balance between the cytokine and its soluble inhibitors. IL-1β was selected as prototypical marker of immunostimulation and inflammation, because of its key role both in inducing/enhancing adaptive immunity and in innate inflammatory reactions.[30] The response of blood leukocytes to LPS was examined one day before surgery (basal response, hypothetically biased by the patient's immunobiography), 1-3 days and 3 months after surgery. Before surgery, patients were classified as normal, anergic (low response to LPS), hyperreactive (excessive reaction to LPS) and inflamed (high baseline activity). Three months after surgery, the innate immune profile variably showed normalization, maintenance of the original profile or adoption of an abnormal profile.

We hypothesized that major surgery induces long-lasting functional redirectioning of innate immune responses in elderly patients, consistent with innate immune memory. Specifically, we postulated that functional leukocyte reactivity to inflammatory challenge may recover or normalize independently of circulating inflammatory biomarkers, reflecting distinct layers of immune adaptation. Furthermore, we aimed to identify clinical and biochemical factors associated with immune trajectories after surgery, with a particular focus on age as a determinant of immune plasticity versus maladaptive immune responses.

MATERIALS AND METHODS

Study design

This study is based on the EU-funded multi-centre prospective observational cohort study "Biomarker Development for Postoperative Cognitive Impairment in the Elderly" (BioCog, https://www. biocog.eu). Recruitment took place from October 2014 to September 2019 at three study sites, two in Berlin, Germany, and one in Utrecht, The Netherlands. The study was approved by the local ethics committees (No. EA2/092/14 in Berlin, and No. 14–469 in Utrecht) and registered on clinicaltrials.gov (NCT02265263). Written informed consent was obtained. The study focused on older, non-demented patients scheduled for major surgery. Eligibility criteria included age of 65 and older and an anticipated surgery duration of >60 min. Additional information on inclusion and exclusion criteria can be found at clinicaltrials.gov.

The study design implied blood collection one day before surgery (T1), 1-3 days after surgery (T2), and 3-4 months later (T3). Of 1033 patients with informed consent, 102 that successfully completed the blood sampling were included in the present analysis, 73 from Berlin and 29 from Utrecht, 31 females and 71 males, from 65 to 84 years of age. Demographic information, clinical data, hematological parameters and other data are reported in Tables S1 & S2A-B.

Whole Blood Assay for evaluation of innate immune reactivity

Blood was collected by venipuncture and anticoagulated with EDTA. Blood aliquots of 250 µL were added to 1.5 mL cryotubes containing 750 µL RPMI-1640 Glutamax medium (GIBCO - Thermo Fisher Scientific, Grand Island, NY, USA) alone (S0) or containing bacterial lipopolysaccharide (LPS, from *E. coli* O55:B5, # L6529, purified by gel filtration, containing <3.0% bacterial proteins and nucleic acids; Merck Sigma-Aldrich, St. Louis, MO, USA), to the final concentrations of 0.025, 0.25, and 2.5 ng/mL blood (S1, S2 and S3). Tubes were tightly capped and incubated at 37°C for 24 h, then additioned with 100 µL 10% Triton-X100 and frozen at -80°C until assay. LPS concentrations were selected, based on preliminary dose-response experiments, to induce a minimal, intermediate, and optimal innate reactivity (on average). It should be noted that, given the high sensitivity of human cells to LPS, the plateau response could be already reached, in several individuals, at the intermediate concentration.

ELISA assay for cytokine and soluble receptor evaluation

Three innate immune factors were evaluated in the blood samples, the inflammatory cytokine IL-1β and the two major natural IL-1 inhibitors, IL-1Ra and the soluble form of the receptor IL-1R2 (sIL-1R2). Samples were thawed, centrifuged and tested for the presence of IL-1β, IL-1Ra and sIL-1R2 with commercial single ELISA Quantikine assays (R&D Systems - BioTechne, Minneapolis, MN, USA). Additional details are reported in the Supplemental Method 1.

Data are expressed as pg/mL blood, which is a representation of innate reactivity at the system level. We have also calculated them as cytokine production per million monocytes (which in blood are the main IL-1 producing cells), to highlight intrinsic cell-based changes. However, since monocyte numbers did not change substantially between pre- and post-surgery times (T1 vs. T3, Table S2B), the data expressed per million monocytes overlap those per mL blood (not shown). Only data per mL blood are reported here, given that they essentially reflect the intrinsic cell-based reactivity data, because not all the cell counts at all time points were available.

Calculation of free active IL-1β and reactivity index

After assessing the IL-1β and sIL-1R2 concentrations in each sample, we have calculated the concentration of free IL-1β (*i.e.*, the fraction of the cytokine not bound to its inhibitor sIL-1R2), as previously described in detail.[31] Briefly, the law of mass action (K_d = ([Ligand] • [Receptor]) / [Ligand • Receptor]) was applied by setting the following parameters: molecular mass of IL-1β, 17 kDa; molecular mass of sIL-1R2, 47 kDa; ratio of IL-1β and sIL-1R2 interaction, 1:1; K_d of IL-1β and sIL-1R2 interaction, 2.7 nM;[32] and the free IL-1β concentration (not blocked by sIL-1R2) was calculated according to the Clark's theory (one ligand, one receptor, specific binding). Thus,

$$[\text{IL-1}\,\beta\,]_{free} = (-[R_T] + [L_T] - K_d + \text{sqr}(([R_T] - [L_T] + K_d)^2 + 4[L_T]\bullet K_d))/2$$

where L_T is [IL-1β], R_T is [sIL-1R2], and K_d is the dissociation constant.

For assessing the fraction of free active IL-1β, the ratio between free IL-1β and the receptor antagonist IL-1Ra was calculated as follows: ($[\text{IL-1}\beta]_{free}$ / [IL-1Ra]) • 1000. Multiplying the ratio by 1000 was performed in view of the notion that, despite the affinity of IL-1β and IL-1Ra for the activating receptor IL-1R1 is similar, it takes about 1000x excess IL-1Ra for an effective inhibition of IL-1β activity. The free active IL-1β value was taken as reactivity index, *i.e.*, a quantitative index representing the innate immune reactivity of blood leukocytes. When IL-1β production was below threshold in the ELISA assay (lower limit of the standard curve 3.9 pg/mL, sensitivity 1 pg/mL), the value was arbitrarily set as 0.1 pg/mL blood. The reliability and robustness of the reactivity index was assessed and validated statistically, as described in the Supplemental Method 2 and Table S3.

Patient grouping

Patients were classified into four functional response categories based on their innate immune reactivity profiles, using AI-assisted methodology (Claude Sonnet 4; Anthropic, San Francisco, CA, USA) as described in detail elsewhere.[33] The classification was based on the reactivity index in response to increasing concentrations of LPS (S1-S3), considering both baseline activity (spontaneous production in the absence of LPS, S0) and dose-dependent response to LPS stimulation.

The four groups — Normal (n=53), Anergic (n=18), Hyperreactive (n=18), and Inflamed (n=13) — were defined by reactivity index criteria summarized in Table 1 (see also Supplementary Methods).

Group assignment was performed independently at T1 (before surgery) and T3 (3 months after surgery), to assess surgery-induced changes in innate immune profiles. Transitions between groups were analyzed to identify patterns of normalization (progression toward Normal), maintenance of the original profile, or development of anomalous reactivity. Full description is provided in Supplemental Method 3. Longitudinal trajectories and distribution analysis of the reactivity indexes at S0, S2, S3 are shown for all patients in the Figure S1 and summarized in Tables S4 and S5. The robustness of the classification was confirmed with a post-hoc sensitivity analysis, varying the

Normal group S3 boundary by ±15% and ±25% (four alternative scenarios) (Table S6).

Statistical analysis

Statistical analyses were performed using Python (version 3.x) with scipy.stats library. Data are presented as median (min-max) or mean ± standard deviation (SD), as appropriate. A p value <0.05 was considered statistically significant. Non-parametric tests were used throughout: Kruskal-Wallis test for multi-group comparisons, Mann-Whitney U test for pairwise comparisons, Wilcoxon signed-rank test for paired T1–T3 comparisons, and χ^2 or Fisher's exact test for categorical variables. Missing data were handled by pairwise deletion (available case analysis), with sample sizes reported per analysis. Full details of the statistical analysis plan, including variable lists, test selection rationale, correction approach, and subgroup exclusion criteria, are reported in Supplemental Method 4.

RESULTS AND DISCUSSION

Patient grouping based on their immune functional states

Out of a cohort of 1033 elderly patients undergoing surgery, within the EU-funded multi-centre prospective observational cohort study "Biomarker Development for Postoperative Cognitive Impairment in the Elderly" (BioCog, https://www. biocog.eu), 102 patients were tested for immune reactivity of their blood leukocytes at 3 time points, one day before surgery (T1), 1-3 days after surgery (T2) and 3-4 months after surgery (T3). At every time point, clinical, biochemical and hematological parameters were also measured (Tables S1 & S2A-B).

Immune reactivity was evaluated by challenging anti-coagulated whole blood with increasing concentrations of bacterial LPS for 24 h, and measuring the production of the inflammatory/immunostimulatory cytokine IL-1β in parallel with its natural inhibitors sIL-1R2 and IL-1Ra. Free active IL-1β, *i.e.,* the proportion of the cytokine that is not neutralized by the sIL-1R2 and not antagonized by IL-1Ra, was calculated and taken as index of immune reactivity. The example in Figure 1 shows the results obtained from a single patient, and includes the quantitative evaluation of IL-1β, sIL-1R2, IL-1Ra, the profile of free IL-1β (not blocked by sIL-1R2) and the reactivity index (*i.e.,* the free active IL-1β calculated as the ratio between free IL-1β and the receptor antagonist IL-1Ra) at T1, T2 and T3. Reactivity indexes obtained from all 102 patients are reported in the Table S4.

Before surgery, the majority of patients showed very low/undetectable basal levels of free active IL-1β, as expected in normal healthy conditions, while others had measurable basal levels, suggesting the presence of a chronic inflammatory status (Table S4). In response to LPS, there was a variable reaction, with some patients showing a dose-dependent increase, others showing an excessive reaction, and others showing partial or substantial refractoriness. Based on their baseline reactivity index and their response to increasing LPS concentrations, patients were classified into four groups. The grouping methodology implied an AI-human collaboration performed in parallel with an analysis based on expert intuition, as previously described,[33] which were merged and additionally optimized upon iterative refinement. Grouping parameters are summarized in Table 1, were validated statistically (Table S3), and parametrized by comparison with a sample of adult donors, in terms of spontaneous and LPS-induced production of IL-1β (higher in response to LPS in adult donors) and IL-1Ra (lower at baseline and in response to LPS in adult donors) (not shown). The group "Normal" includes 53 patients whose blood cells were quiescent at baseline (S0) and responded to LPS stimulation (S1-S3). In this group, the peak response to LPS varied between S2 and S3. The group "Anergic" (18 patients) includes patients that were quiescent at baseline, but showed low reactivity to all LPS concentrations, suggestive of impaired capacity to react to stimuli, either due to immunosenescence or to compensatory lower functionality (as expected from monocytes biased by an inflammatory microenvironment). The group "Hyperreactive" (18 patients) includes patients with an excessive reaction to all LPS concentrations, suggestive of hyperreactivity to stimuli. Eventually, the group "Inflamed" (13 patients) showed a high activation level at baseline, suggestive of ongoing inflammation. Immune reactivity indexes are listed in Table S4, while patients' characteristics are reported in Table S7.

Immune transition to normalization after surgery

As expected, immediately after surgery (T2), the majority of patients showed an evident decrease/refractoriness in the reactivity to LPS (post-surgery immunosuppression). It was however notable that the reactivity index was higher in about 10% of patients, suggesting a transient surgery-induced inflammation (Table S4).

While the majority of Normal patients (71.7%) maintained their normal profile at T3, the transition from T1 to T3 shows a substantial degree of immune normalization in Anergic and Hyperreactive patients: 10/18 Anergic patients (55.6%) and 12/18 Hyperreactive patients (66.7%) normalized at T3 (Table 2). Eventually, only two of the 13 Inflamed patients normalized at T3, while 9 remained Inflamed and two became Hyperreactive. Overall, 62/102 patients showed normal immune reactivity (60.8%) 3 months after surgery, including 24 patients that have normalized their previously anomalous reactivity (49.0%), while 24.5% patients maintained anomalous reactivity and 14.7% adopted an abnormal profile. The data are shown in Figure 2, Table 2 and Tables S8-S9.

By examining the parameters leading to immune normalization at T3, for patients in the Anergic group this was mainly due to an increase of IL-1β production, with either unchanged or decreased IL-1Ra (Figure 3, Table S8, Figure S2). For Hyperreactive patients, normalization was mainly due to an increase in IL-1Ra production accompanied, in some cases, with a decrease in IL-1β production (Figure 3, Table S8). For the two Inflamed patients that normalized at T3, this was due to a substantial decrease of the spontaneous IL-1β production (Figure 3, Table S8). For Normal patients that remained Normal, it is interesting to see that their responsiveness at T3, in terms of mediator production, can be different from that observed at T1, with some patients showing a concomitant increase or a concomitant decrease of IL-1β and IL-1Ra production, and other showing variations in the production of the two mediators that however remained within the Normal range (Figure 3, Table S8). In general, variations in the sIL-1R2 levels were minimal and never significantly affected the overall variations of the reactivity index (not shown). This suggests that attaining normal responsiveness, but also maintaining it, is an active mechanism for improving defensive capacities, upon adaptation to the incurring physical and biological challenges.

Immune transition to abnormality after surgery

About 40% of patients did not attain immune normalization after surgery. Of the 15/53 Normal patients that showed abnormal immunity 3 months after surgery, 10 showed decreased responsiveness, 4 showed hyperreactivity, and only 1 became inflamed (Table S9).

Following the concept that monocytes/macrophages in the elderly modify their functions as a compensatory mechanism to tackle the changes in the aged body, we may hypothesize that adoption of an Anergic profile is a hypo-compensation to control excessive inflammation and prevent damage, while adopting an Hyperreactive profile could be a hyper-compensation for ensuring protection in the absence of a functional adaptive immunity. The same could be said for Anergic and Hyperreactive patients that maintained their original profile. A case apart seems to be that of Inflamed patients. The vast majority maintains the abnormal Inflamed profile at T3, suggesting a substantial refractoriness to adaptation, i.e., a senescent irreversible status of pathological inflammation.

In the case of lack of normalization, the abnormal reactivity at T3 was also due to changes in the production of IL-1β and IL-1Ra: decreased IL-1β in most patients transitioning to Anergic at T3, higher IL-1β or lower IL-1Ra for those transitioning to Hyperreactive, and increased baseline IL-1β for those transitioning to Inflamed (Table S9).

Immune trajectory prediction by pre-surgery biomarkers

Correlation of the functional response groups with biochemical/hematological parameters pre-surgery was assessed, with only five out of 32 biomarkers showing significant associations with group assignment, i.e., sSORL1, neutrophil counts, zonulin, glucose and IL-6 (Supplemental Results, Figure S3, Table S10).

Functional immune transitions were only partially accompanied by biochemical/hematological profile conversion (Tables S11-S12). Patients who remained stably Normal (Normal→Normal, n=38) maintained their char-

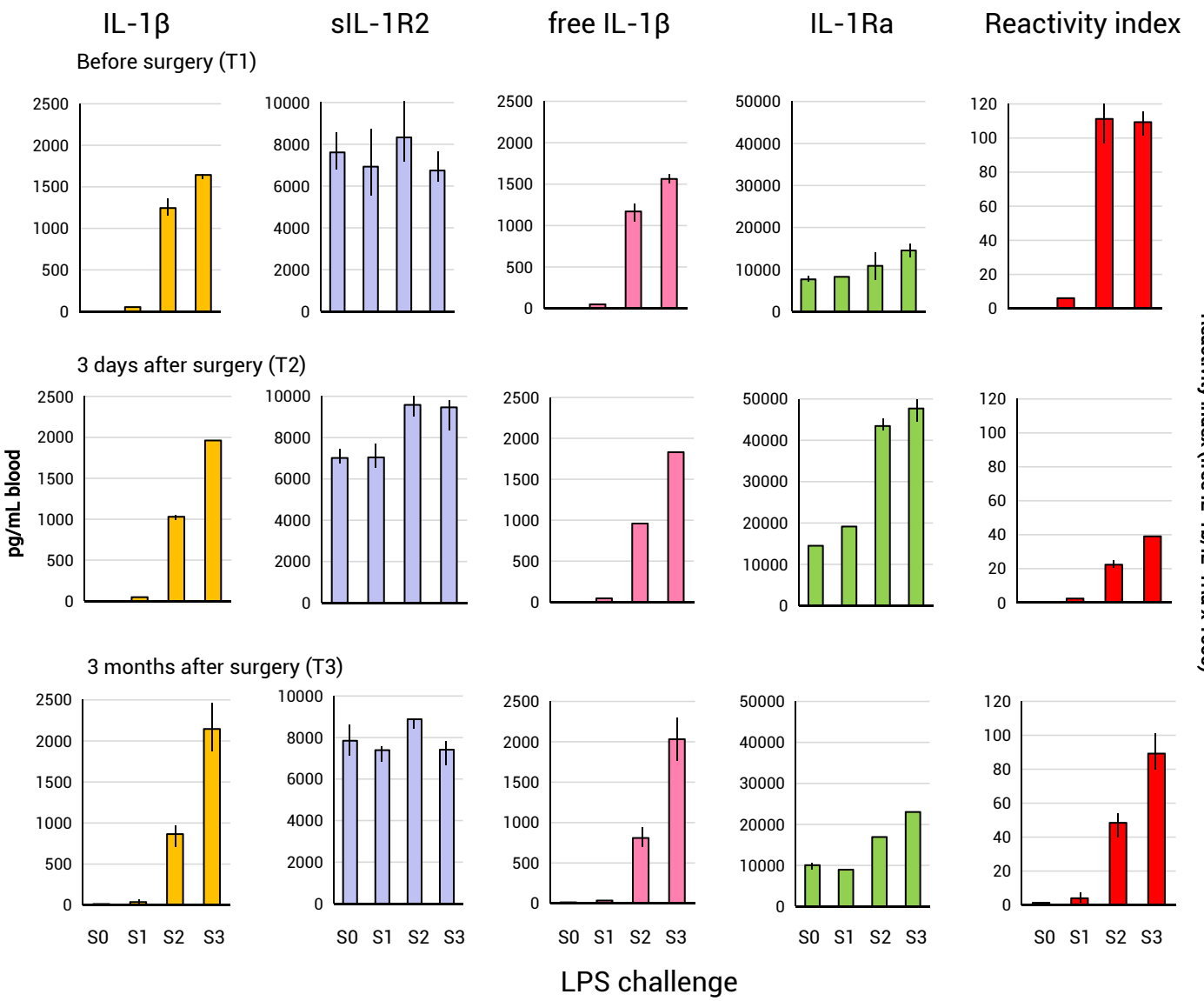

Figure 1. Calculation of the immune reactivity index Data are shown from a single patient before surgery (T1; first line), 3 days after surgery (T2; second line) and 3 months later (T3; third line). Blood cells were exposed for 24 h to culture medium alone (S0) or to 0.025, 0.25 and 2.5 ng/mL LPS (S1, S2, S3), and tested for the production of IL-1β (first column from left), sIL-R2 (second column) and IL-1Ra (fourth column). The concentration of free IL-1β (third column) was calculated according to the law of mass action based on the 1:1 interaction between IL-1β and sIL-1R2. Eventually, the immune reactivity index (fifth column) was calculated as the ratio between free IL-1β and IL-1Ra multiplied by 1000 (see Materials and Methods). Data are presented as mean ± SD of duplicate assessments.

acteristic biochemical profile, with T3 values closely matching their T1 reference profile for all five discriminating biomarkers. In contrast, patients transitioning from Normal to abnormal states showed only partial acquisition of their destination group's biochemical/hematological signature. Similarly, patients transitioning to Normal achieved functional immune normalization without complete biochemical profile conversion (Table S11). This suggests that immune and biochemical changes either occur at different temporal scales or are independent.

Investigation of the possible predictive value of the circulating biomarkers shows that none of the five biomarkers at T1 could predict trajectory outcome (all $p>0.05$) (data not shown). This lack of predictive value extended to all 32 circulating biomarkers tested (0/32 significant, 0% success rate), demonstrating that circulating biochemical/hematological profiles, while descriptive of current immune state, do not forecast future trajectories.

Systematic analysis of 29 clinical parameters revealed that age was a significant predictor of trajectory in Normal patients ($p=0.015$, Kruskal-Wallis test), but not for other groups (Table 3). Normal patients who transitioned to Anergic at T3 were significantly older (median 76 years, range 66-84) compared to those who remained stable (median 72 years, range 65-84) or shifted to Hyperreactive (median 66 years, range 65-68). This pattern suggests age-dependent divergent immune responses to surgical stress: young-old patients (65-70 years) showing residual immune reactivity leading to hyper-compensation, middle-olds (70-75 years) maintaining optimal balance, and old-old patients (>75 years) adopting immune hypo-compensation/anti-inflammation. Thus, age represents a robust, clinically applicable predictor of post-surgical immune trajectory. Surgery/anesthesia duration showed a borderline association with trajectory ($p=0.063$), with longer procedures potentially associated with a shift toward Hyperreactive state (Table 3). Similar analyses for Anergic, Hyperreactive, and Inflamed patients at T1 revealed no significant predictors among major clinical parameters (age, BMI, comorbidities: all $p>0.10$). No correlation was found between trajectories and other major clinical parameters (BMI, comorbidities, site of surgery, pharmacological treatment, etc.; all $p>0.10$) in any group. Potential associations were only identified with some less common factors: stroke/TIA history in Anergic patients who normalized ($p=0.009$, n=3), post-operative pain in Anergic patients who remained Anergic ($p=0.021$, n=2), and current smoking in Hyperreactive patients who normalized ($p=0.017$, n=2) (data not shown). However, these associations are based on extremely small subgroup

Table 1. Classification of patients based on their innate immune reactivity before surgery.

Group	Stimulus*	Criteria (reactivity index)@	Average reactivity index$	SD$	Range$
Normal	nothing	≤5	0.6	1.5	0.0 – 5.7
	LPS 0.01	≤10	2.0	2.8	0.0 – 9.7
	LPS 0.1	>S1	25.6	16.6	2.7 – 70.6
	LPS 1.0	≥20 <75	40.8	16.8	19.2 – 74.8
Anergic	nothing	≤5	0.6	1.3	0.0 – 3.8
	LPS 0.01	≤20	2.7	6.4	0.0 – 27.0
	LPS 0.1	≤20	6.6	4.6	0.0 – 15.8
	LPS 1.0	≤20	10.9	5.2	0.0 – 19.8
Hyperreactive	nothing	≤5	0.2	3.2	0.0 – 3.3
	LPS 0.01	0-40	8.1	3.8	0.0 – 36.7
	LPS 0.1	>S1	63.8	31.7	12.1 – 105.8
	LPS 1.0	>S2	108.9	35.7	64.8 – 219.1
Inflamed	nothing	≥10	24.0	18.2	9.4 – 107.6
	LPS 0.01	variable	20.2	17.6	2.4 – 83.8
	LPS 0.1	variable	43.1	23.9	7.5 – 102.4
	LPS 1.0	variable	43.4	31.6	13.5 – 89.7

* LPS concentrations are given as ng/mL blood in the *in vitro* test.

@ Grouping criteria, based on the reactivity index, *i.e.*, ([IL-1β_{free}]/[IL-1Ra])*1000.

$ Averages, SD and range of the reactivity indexes were calculated for patients included in the four groups: 53 patients in group Normal, 18 in Anergic, 18 in Hyperreactive and 13 in Inflamed (see Table S3 for additional information).

Table 2. Patients' immune reactivity transition from T1 to T3.

Cluster at T1	N. patients at T1 (%)	N. patients transitioning to different groups at T3 (% of T1)			
		Normal	Anergic	Hyperreactive	Inflamed
Normal	53 (52.0)	38 (71.7)	10 (18.9)	4 (7.50)	1 (1.90)
Anergic	18 (17.6)	10 (55.5)	7 (38.9)	1 (5.55)	0 (0.00)
Hyperreactive	18 (17.6)	12 (66.7)	1 (5.55)	4 (22.2)	1 (5.55)
Inflamed	13 (12.7)	2 (15.4)	0 (0.00)	2 (15.4)	9 (69.2)
N. patients at T3 (%)		*62 (60.8)*	*18 (17.6)*	*11 (10.8)*	*11 (10.8)*

sizes and require validation in larger independent cohorts before clinical application.

CONCLUSION

We have assessed in this study the changes in blood innate immune reactivity of elderly patients undergoing massive surgery. The goal was to explore the possibility that changes in immune reactivity could be due to the establishment of an innate memory at the level of blood leukocytes.

Innate immune reactivity was assessed as production of free active IL-1β by blood leukocytes in response to a prototypical bacterial challenge (LPS from Gram-negative bacteria). Although not reproducing the global immune reactivity of human subjects, this assay can provide a realistic picture of the responsiveness to bacterial infections. The choice of LPS as microbial challenge allows for a good assessment of innate reactivity, since LPS can activate innate cells through several receptors/sensors, besides the typical TLR4 complex, and activate both anti-bacterial and anti-viral responses.[34-36] Also, we have used an LPS preparation that intentionally contains small amounts of bacterial proteins and nucleic acids, so that other pattern-recognition receptors can be activated. The choice of IL-1β as endpoint of immune activation also allows for extending the immune assessment to adaptive immunity, since IL-1β is a key factor required for initiating and amplifying adaptive immune responses at several levels, from antibody production to development of CTL and Th17 cells.[30,37-39] Thus, the production of IL-1β implies the ability of mounting an adequate adaptive immune response. We have included in the assessment the balance between IL-1β and its natural inhibitors, which are also produced in response to LPS (or any other microbial stimulus), so that we can provide the measure of the free active IL-1β fraction, as a more reliable endpoint of functional immune reactivity. Thus, despite its limitations (only one microbial stimulus, only one immune endpoint), the adopted assay should provide a good picture of immune reactivity that, although based on an innate response, can extend to adaptive immune competence.

Notably, the observed changes at the system level (production of IL-1β, IL-1Ra and sIL-1R2 per milliliter of blood) were largely cell-intrinsic, as the changes relative to monocytes were fully overlapping with those relative to whole blood. We could therefore confidently hypothesize that the changes observed could be based on the surgery-induced modulation of the intrinsic monocyte reactivity, likely to the establishment of innate memory. That innate memory can be generated by traumatic events, beyond infectious agents, is expected, as its development was reported after brain damage (stroke),[40] heart failure,[41] organ transplantation[42] and chronic stress,[43] possibly initiated by release of DAMPs (damage-associated molecular patterns) and stress-

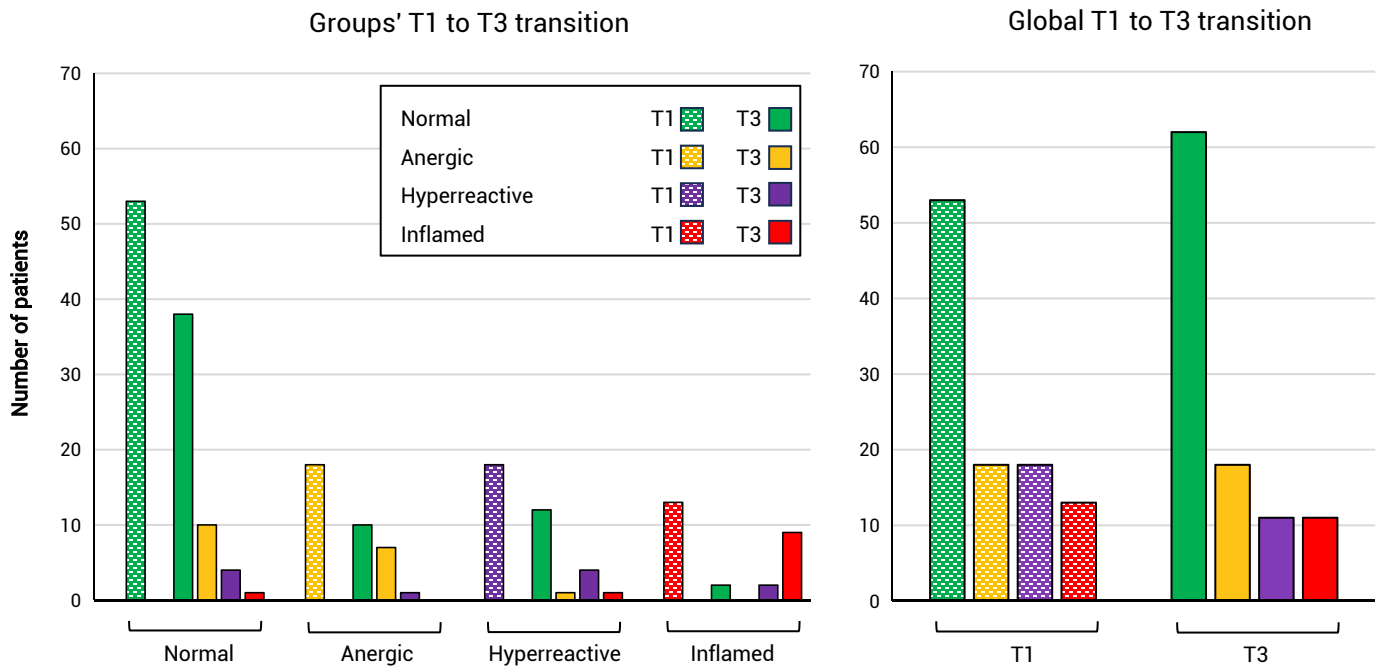

Figure 2. Immune reactivity transition from T1 to T3 Left panel: Immune reactivity transition from T1 to T3 is represented for the four distinct groups. Patients are distributed in four groups at T1 (dotted columns). Normal, green; Anergic, orange; Hyperreactive, violet; Inflamed, red. Their immune reactivity transition from a group to another at T3 is shown as solid color columns. Right panel: Global immune reactivity transition from T1 to T3 is represented for the entire patients' cohort. Dotted columns: reactivity at T1, solid columns, reactivity at T3.

stimulated neurotransmitters/hormones. The putative memory-inducing event in this study is surgery, which however intersects with a memory profile already shaped by multiple life-long challenges experienced by the patients and with the aged tissue microenvironmental cues. We have excluded the influence of other co-occurring circumstances before surgery (pharmacological treatments, presence of tumors, comorbidities, type of surgery) on the immune changes observed after surgery. While other explanations are definitely possible, the evidence obtained here does not contradict the possibility that the immune changes observed after surgery are due to immune cell reprogramming, combined with adaptation to the surgery-dependent changes in the body microenvironmental conditions. Thus, our goal was to examine whether a new event (surgery) could modulate the pre-existing innate immune memory of elderly people. Elderly people have in general a high background inflammation ("inflammaging") caused by increased frequency of cell death and molecular/metabolic anomalies,[24,25] in parallel with senescence of adaptive immunity. In this study, we have taken the production of free active IL-1β as index of immune reactivity, a choice that intends to encompass both innate immunity/inflammation and adaptive immunity, since IL-1β is a major inflammatory cytokine in mammals but it is also a main factor in driving adaptive immune activation.[30,37-39] The study shows that only a limited number of patients displayed significant constitutive inflammation (13/102, group Inflamed), suggesting that, despite the old age, there are mechanisms that compensate the anomalous inflammatory tissue environment and control the development of chronic inflammation. On the other hand, several patients showed abnormal response to bacterial stimulation, with 18/102 being hyporeactive (group Anergic) and 18/102 being hyperreactive (group Hyperreactive), suggesting either inadequate capacity to tackle inflammatory/infectious challenges, or the implementation of compensating mechanisms. In the case of Anergic patients, a hypo-compensating mechanism is a likely hypothesis, in particular for monocyte/macrophages that, in elderly people, are biased by the inflammatory microenvironment towards more active anti-inflammation and repair functions paralleled by a much lower reactivity to inflammatory cues. In the case of Hyperreactive patients, the increased monocyte reactivity could be due to a hyper-compensating function, aiming at replacing an inadequate responsiveness of the adaptive immune system. Notably, over 50% of patients could be considered normal, with no background inflammation and with dose-dependent reactivity to stimulation.

The results show that the majority of patients with a pre-surgical "Normal" immune profile maintain their optimal reactivity 3 months after surgery. It is very interesting that functional maintenance often implies active changes in immune responsiveness, with concomitant decreases or increases in the levels of IL-1β and its main inhibitor IL-1Ra observed after surgery. In patients with an initial abnormal immune profile (Anergic, Hyperreactive, Inflamed), immune normalization at T3 depended on substantial changes in the levels of IL-1β and IL-1Ra, which allowed to reach a balanced equilibrium with consequent optimization of the reactivity. Likewise, in the case of patients that did not normalize, the anomalous reactivity after surgery also depended on variations in the mutual levels of the two factors. Thus, in all cases the post-surgery reactivity was the result of a remodulation of immune reactivity.

Neither the pre-surgery reactivity indexes nor the circulating inflammation-related hematological/biochemical parameters could allow us to predict the post-surgical immune trajectory. While five of these biomarkers (sSORL1, zonulin, neutrophils, IL-6 and glucose) were significantly associated with the initial immune reactivity profiles, none of them could predict the post-surgical trajectory. Post-surgical immune normalization did not correlate with biomarker normalization, suggesting that functional immune normalization is independent of hematological, biochemical or metabolic normalization. The only parameter that significantly correlated with a pathological trajectory was age for Normal patients, with the older patients having significantly higher chances to develop post-surgical anergy. This suggests an age-dependent increase in the body inflammation that biases innate immune cells towards anti-inflammation.

Overall, the most impressive observation in this study is that the majority of elderly patients show excellent innate immune reactivity, and plasticity in adapting to external stresses by remodulating their responsiveness. The immune inertia and hyperreactivity shown by a percentage of patients seem to depend on the previous history of exposure to external and internal challenges (diseases, infections, etc.), ongoing "inflammaging" and adaptive immune dysfunctions, rather than on an intrinsic and irreversible cell senescence. This is suggested by the fact that they can recover optimal reactivity upon a given challenge (10/18 Anergic and 12/18 Hyperreactive patients became Normal at T3). This evidence supports the hypothesis that elderly

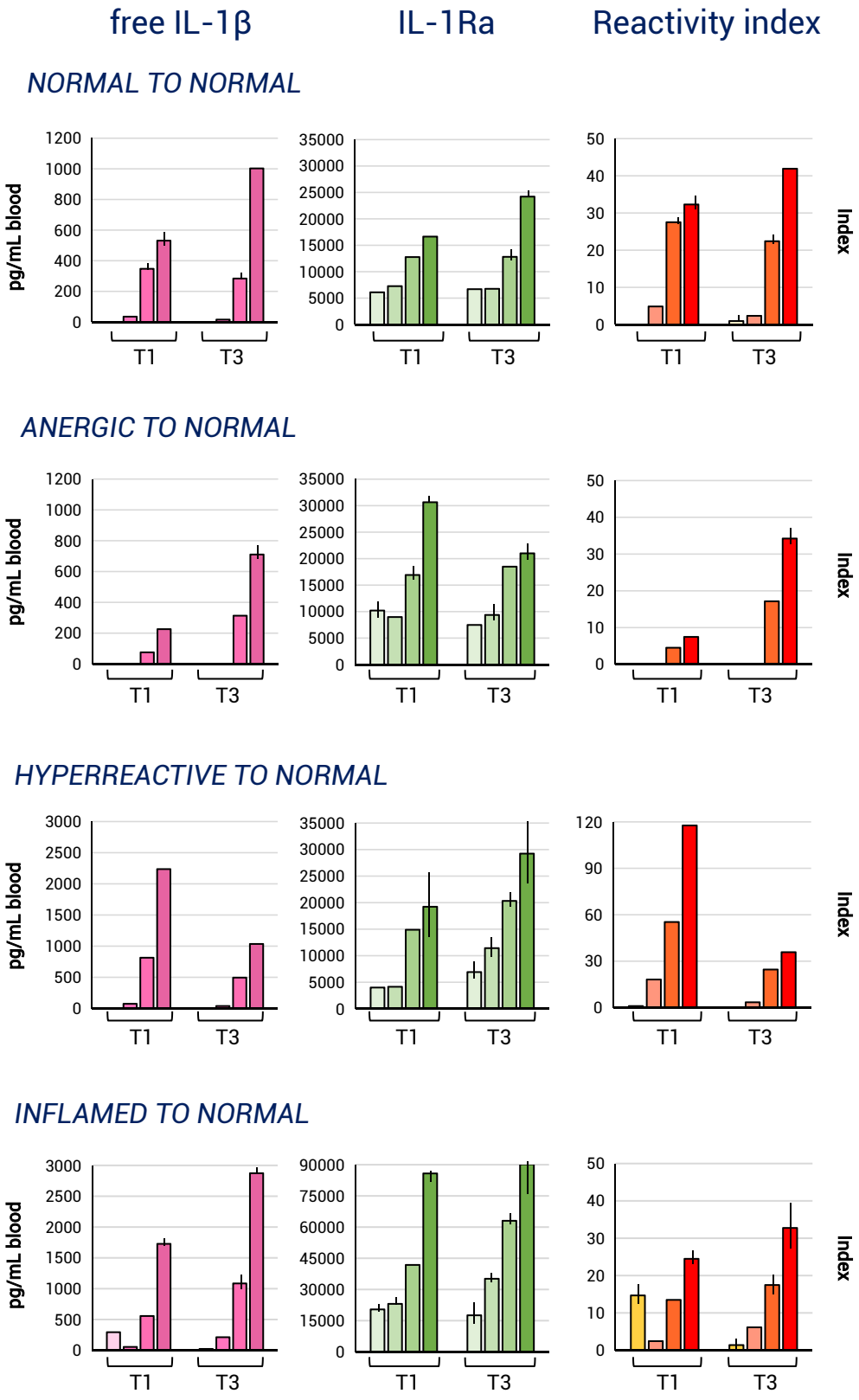

Figure 3. Modalities of immune reactivity normalization at T3 Data are from individual patients, taken as examples. Line *NORMAL TO NORMAL* shows that concomitant upregulation of free IL-1β (left column; purple) and IL-1Ra (center column; green) results in a lack of change in the reactivity index at T3 (left column; red). Line *ANERGIC TO NORMAL* shows that the normalization of the reactivity index at T3 depends on the upregulation of free IL-1β and no change and/or concomitant decrease in IL-1Ra. Line *HYPERREACTIVE TO NORMAL* shows that the normalization of the reactivity index at T3 depends on decrease in free IL-1β and upregulation of IL-1Ra. Please note the different scale for free IL-1β and Reactivity index, compared to the first two cases. Line *INFLAMED TO NORMAL* shows that the normalization of the baseline reactivity index at T3 depends on the substantial downregulation of free IL-1β and no change in the very high production of IL-1Ra. Here as well, please note the higher scale for free IL-1β and IL-1Ra. In each panel, exposure to LPS (S0, S1, S2, S3) is represented by color scaling, from the lightest (S0, no LPS) to the darkest (S3, LPS 2.5 ng/mL). Data are presented as mean ± SD of duplicate assessments.

Table 3. Clinical predictors of immune trajectory in Normal patients at T1.

Parameter	Remained Normal (n=38)	→Anergic (n=10)	→Hyperreactive (n=4)	*p*-value (KW/χ^2)
Age (years)	72.5 ± 5.0	74.7 ± 5.7	66.5 ± 1.3	0.015*
Sex (male, %)	26 (68%)	5 (50%)	4 (100%)	0.190
BMI (kg/m^2)	26.8 ± 4.0	26.7 ± 7.2	27.1 ± 2.0	0.883
Charlson Comorbidity Index	1.0 ± 1.2	1.1 ± 1.1	0.5 ± 1.0	0.594
MMSE score (points)	28.6 ± 1.6	29.2 ± 0.9	29.0 ± 1.2	0.670
ISCED education score	2.1 ± 0.7	2.4 ± 0.7	3.0 ± 0.0	0.058
Arterial hypertension (%)	23 (61%)	5 (50%)	1 (25%)	0.364
Diabetes (%)	3 (8%)	2 (20%)	0 (0%)	0.407
History of stroke/TIA (%)	3 (8%)	1 (10%)	0 (0%)	0.814
Current smoker (%)	7 (18%)	0 (0%)	0 (0%)	0.482
Surgery duration (min)	173 ± 121	138 ± 129	329 ± 147	0.063

Values are mean ± SD for continuous variables or n (%) for categorical variables. Kruskal-Wallis test (KW) for continuous variables; Chi-square test (χ^2) for categorical variables. *$p<0.05$.

The parameters shown in the table were selected to represent major clinical domains (demographics, anthropometrics, comorbidities, cognition, lifestyle factors, surgical characteristics). Other 18 clinical parameters tested (including height, weight, MNA score, ADL scores, TUG test, days in hospital, ASA status, premedication, benzodiazepine use, tumor presence, previous surgeries, preoperative cognitive impairment, site of surgery): all $p>0.10$.

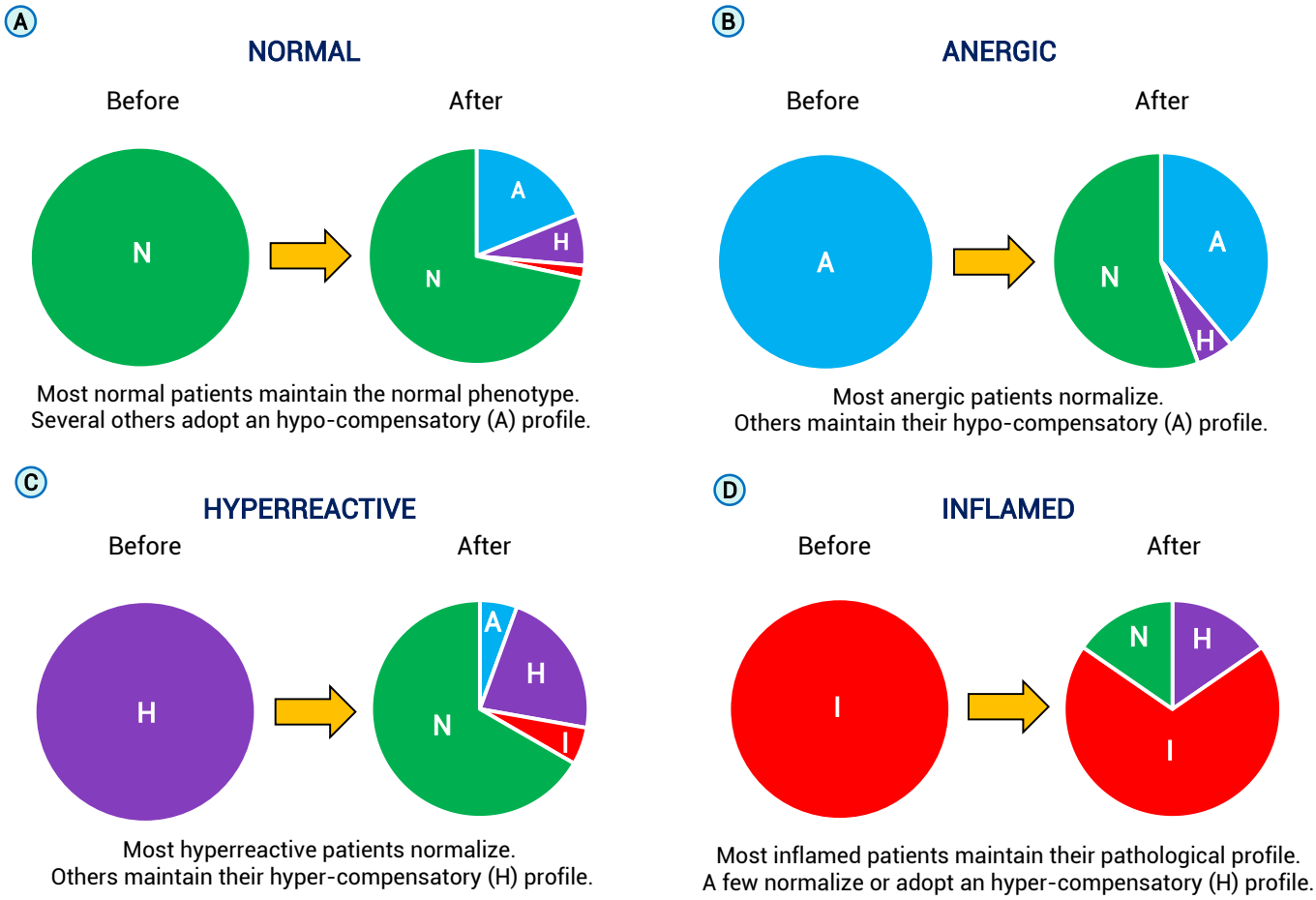

Figure 4. Schematic representation of the immune responsiveness grouping of patients and their transition after surgery The scheme represents the immune transitions of elderly patients after surgery. A. Patients functionally classified as "Normal" (N, green) before surgery largely maintain their normal profile after surgery. However, some of them transition to the abnormal compensatory profile of anergy (A, blue) and a few to hyperreactivity (H, purple). Only one transitions to the more pathological Inflamed profile (red). B. Patients initially classified as "Anergic" (A, blue) mostly transition to a normal N profile or maintain their anergic A functions. Only a few adopt the hyperreactive H profile. C. Initially Hyperreactive patients (H, purple) largely transition to normal N after surgery, while some maintain the initial hyperreactivity H, and very few transition to anergic A and inflamed (I, red) profiles. D. Inflamed I patients largely maintain their abnormal profile after surgery, suggesting the possibility of an irreversible senescent status. However, some patients adopt a normal N profile and others transition to hyperreactivity H, suggesting a reversible functional status.

individuals may retain functional innate immunity and the capacity to fight diseases, despite the immunosenescence and exhaustion of T and B cells and the overall decline of adaptive immunity. Thus, we propose that the "aging" of innate immunity in the elderly[44] is not an intrinsic defect due to immunosenescence, but it is a "compensatory" anomalous functional phenotype mainly due to the individual immunobiography, i.e., accumulation of past experiences that bias the next responses, which aims to adapt to the changes in the aging body. Thus, we can hypothesize that such biases are reversible and can be modified.[26–29] Figure 4 summarizes the findings on which our hypothesis is based. This notion opens the way to future interventions targeting innate immune memory aiming to improve immune competence in the elderly, thereby strengthening their resistance to infections and diseases, as an important component of immune rejuvenation strategies.

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AUTHOR CONTRIBUTIONS

DB conceived the work, critically analyzed the data, and wrote the manuscript. MLM defined the AI-assisted patient clustering. PM and DB refined the AI-assistant clustering by contributing basic and clinical immunological expertise. MLM performed the statistical analysis of data. PM and MLM critically revised the data and contributed to writing the manuscript. PI coordinated the immunological experimental activities, collected the data, and critically revised the manuscript. DM and AM organized and conducted the experimental activities. GW, TBD and AT coordinated the international work. CDS coordinated the patients' recruitment, demographic data collection, blood collection and evaluation of the biochemical parameters, together with the entire BioCog consortium. All authors contributed to and approved the manuscript.

DECLARATION OF INTERESTS

Diana Boraschi serves as co-Editor-in-Chief of The Innovation Life and was blinded from reviewing or making final decisions on the manuscript. Peer review was handled independently of this member and their research group. The other authors declare no competing interests.

ETHICAL STATEMENT AND PATIENT CONSENT

This study represents a retrospective analysis of anonymized data previously collected as part of the BioCog consortium study ("Biomarker Development for Postoperative Cognitive Impairment in the Elderly", grant n. 602461/HEALTH-F2-2014-60246). All original data collection procedures were performed in compliance with relevant laws and institutional guidelines and were approved by the local medical ethics committees of the study centers in Berlin, Germany (EA2/092/14) and Utrecht, The Netherlands (14–469). Informed consent was obtained from all participants for the original data collection. Patient privacy rights were strictly observed throughout the data handling process. As this current analysis involved only retrospective evaluation of fully anonymized datasets, no additional ethics approval was required.

DATA AND CODE AVAILABILITY

The datasets used in this study are part of the BioCog study. Due to patient privacy constraints and consortium agreements, the raw datasets that are not presented here are not yet publicly available. Before their publication, access to anonymized data may be possible through the BioCog consortium for qualified researchers with appropriate institutional approvals and data use agreements. Requests for data access should be directed to the BioCog consortium leadership (Georg Winterer, Thomas B. Dschietzig and Claudia Spies).

SUPPLEMENTAL INFORMATION

It can be found online at https://doi.org/10.59717/j.xinn-med.2026.100223.