EXTENDED REPORTS

Effect of IL15 on T cell clonality in vitro and in the synovial fluid of patients with rheumatoid arthritis

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Abstract

Objective—Recent studies have suggested that interleukin (IL) 15 induces T cell accumulation in synovial lesions of rheumatoid arthritis (RA). This study aimed at determining whether this cytokine could explain in vivo T cell clonality in RA.

Methods—Peripheral blood mononuclear cells (PBMC) from patients with RA were stimulated in vitro with IL15 or IL2. After isolation of mRNA from stimulated cells and synovial T cells, genes coding the V-D(N)-J (CDR3) region of T cell receptor β chains were amplified by a reverse transcriptase polymerase chain reaction. A single strand conformation polymorphism analysis was used to detect the clonotype(s) of accumulating T cells. Nucleotide and amino acid sequencing was also performed.

Results—Stimulation of PBMC with IL15 resulted in oligoclonal expansion of T cells. However, IL15 induced clones from PBMC were mostly different from the dominantly expanding T cell clones in synovial fluid. Furthermore, IL15 and IL2 responding clones were only partially identical.

Conclusions—Although IL15 results in clonal accumulation of T cells, T cell clonality in rheumatoid joints could not be explained by the effect of IL15 alone. The results indicated the requirement of other factor(s), in addition to IL15, in the pathological process affecting RA joints. The results also suggested different responses by each T cell clone to IL15 or IL2.

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The precise mechanisms that lead to the development and persistence of arthritic inflammation in patients with rheumatoid arthritis (RA) are still unknown. One such mechanism is thought to be the antigen-specific autoimmune response of T cells in the affected joints. This view is supported by infiltration of synovial inflammatory lesions by T cells (most are CD4+CD45RO+—that is, “memory-type” cells).¹ The reported correlation between distinct HLA haplotype (for example, HLA-DR4) and susceptibility to RA also indicates a

specific interaction between the major histocompatibility complex and T cells. Moreover, it has been reported that T cells accumulating in RA joints are oligoclonal,^{2–5} indicating the presence of antigenic stimulation(s). Thus, T cell lines or clones specific for candidate autoantigens, such as type II collagen, have been established in vitro from patients with RA.⁶ However, because the exact pathogenic (or arthrogenic) antigen(s) has not been identified in vivo, the specific stimuli that induce T cell accumulation in situ are still unclear.

On the other hand, development and persistence of RA might also be due to mechanisms independent of T cells. In this case, synovial cells or cytokines are thought to be the primary disease initiating factors. Administration or gene expression of certain cytokines—for example, interleukin (IL) 1, is reported to produce RA-like arthritic lesions.⁷ Thus, according to this concept, T cell accumulation in RA synovium is explained by the non-specific effect of such cytokines, rather than by the immune response(s) against intra-articular autoantigen(s).

IL15, a 14 to 15 kDa cytokine produced by monocytes/macrophages, was originally identified as a T cell proliferating factor with biological activities similar to those of IL2.⁸ IL15 shares the β and γ chains of the IL2 receptor with IL2, in addition to a unique IL15 receptor α chain (see review⁹). Recently, McInnes and Liew suggested that IL15 is a disease modulating factor in RA.⁹ In fact, overexpression of IL15 in the synovial membrane of patients with RA has already been demonstrated.^{10–11} Furthermore, IL15 could stimulate the induction of tumour necrosis factor α (TNF α) from synovial T cells¹² and acts as a chemoattractant to T cells.⁹ Thus synovial T cells, under the influence of IL15, are thought to exert proinflammatory effects in the absence of specific recognition of local antigens.^{9–10}

To investigate the role of IL15 on T cell clonality in RA we used a T cell clonality analysis system reported previously by Yamamoto *et al.*³ A combination of reverse transcriptase polymerase chain reaction (RT-PCR) and single strand conformation polymorphism (SSCP) analyses was used to detect

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Figure 1 Clonotype analyses of interleukin (IL) 15 stimulated T cells. (A) Peripheral blood lymphocytes (PBL) from two healthy subjects and two patients with rheumatoid arthritis (RA) were stimulated *in vitro* with IL15 and analysed for T cell receptor clonality with a reverse transcriptase polymerase chain reaction and single strand conformation polymorphism (RT-PCR-SSCP). Lanes indicate, from left to right, results of the final SSCP analysis of T cell receptor BV1 to 20 subfamilies, respectively. (B) Results of SSCP analyses of T cells in precultured PBL, in synovial fluid (SF), and in PBL stimulated with either IL2, IL15, or phytohaemagglutinin (PHA) from a patient with RA.

T cell clones that accumulate in response to antigenic stimulation *in vivo* and *in vitro*.^{3 13 14} Using this method, we tested in this study whether IL15 stimulation induced T cell clonal

expansion similar to that seen in RA joints *in vivo*. Our results showed a discordance between the effect of IL15 and T cell clonality of *in vivo* samples. Our findings suggested that IL15 is not a critical cytokine for the induction of T cell clonality in RA joints *in vivo*.

Materials and methods

PATIENTS

Six female patients with definite RA (mean (SD) age 61.0 (9.3), mean disease duration 10.8 (7.5) years), who satisfied the revised criteria of the American College of Rheumatology,¹⁵ were enrolled in this study. Patients had a stable disease and were being treated with non-steroidal anti-inflammatory drugs, prednisolone (5–10 mg daily), and disease modifying antirheumatic drugs (for example, bucillamine or methotrexate, or both) at the time of the study.

CLINICAL SAMPLES

Clinical samples were obtained from the patients with RA as well as from healthy volunteers after informed consent. Synovial fluid (SF) samples were obtained during therapeutic arthrocentesis of the knee joint. Peripheral blood samples were also obtained from the patients at the time of SF sampling. Blood and SF samples were placed in separate tubes containing heparin. Mononuclear cells in the samples were separated by a standard Ficoll-Paque (Pharmacia, Uppsala, Sweden) gradient centrifugation. A proportion of the extracted cells was incubated as described below, while the remaining cells were immediately soaked in RNAzolB solution (Tel-Test, Inc, Friendswood, TX) for RNA isolation.

CELL CULTURE

Peripheral blood mononuclear cells were suspended in RPMI medium supplemented with 10% fetal calf serum, 1% penicillin-streptomycin, and 1% L-glutamine at a concentration of 1.2×10^6 cells/ml. Cells were cultured with or without one of the following reagents for six days: IL2 (at a final concentration of 100 µg/ml; Shionogi and Co, Japan), IL15 (100 ng/ml; Genzyme, Cambridge, MA), or phytohaemagglutinin-P (10 µg/ml; Sigma-Aldrich Japan KK, Tokyo, Japan). After incubation, the cells were harvested, washed, and immediately soaked in RNAzolB solution.

RNA ISOLATION, CDNA SYNTHESIS, AND RT-PCR-SSCP ANALYSIS

T cell clonality was determined by RT-PCR-SSCP as described previously with minor modifications.³⁻⁵ Briefly, mRNA isolated from each sample with RNAzolB solution was converted to first strand cDNA by reverse transcriptase (SuperscriptII, Gibco BRL, Gaithersburg, MD) and random hexamer oligonucleotide priming (100 pmol, Gibco). A similar amount of cDNA from each sample was mixed with each primer set (one of the BV sense primers (BV 1–20)) and a non-biotinylated BC antisense primer; the sequences of these primers have been previously described.^{3 5} A PCR reaction was carried out

Figure 2 Comparison of accumulating T cell clones between cytokine stimulated cells and *in vivo* accumulating cells in the synovial fluid (SF). Amplified DNAs were electrophoresed on the same SSCP gel and the accumulating clonotypes were compared by their difference in CDR3 sequences. PBL = peripheral blood cells; PHA = phytohaemagglutinin.

with a Hybaid thermal reactor (Hybaid, Middlesex, UK) using dNTP and Taq DNA polymerase (TaKaRa Shuzo Co, Shiga, Japan) for 35 cycles (94°C for one minute, 58°C for two minutes, and 72°C for two minutes). Amplified DNA was diluted, heat denatured, and then electrophoresed in non-denaturing 4% polyacrylamide gels. After electrophoresis, DNA was transferred to a nylon membrane (Gene Screen, Biotechnology Systems, NEN Research Products, Boston, MA) and then hybridised with a BC internal probe (the sequence has been described previously⁵). The DNA was finally visualised after incubation with a chemiluminescent system (Phototope-Star detection kit for nucleic acids, New England Biolabs, Beverly, MA).

DNA SEQUENCING

The first PCR products were allowed to proceed directly to a second PCR using BV and

BC specific primers with enzymatic (*EcoRI* or *HindIII*, respectively) sites. After digestion, the amplified DNAs were subcloned into a plasmid vector (pBluescriptII, Stratagene, La Jolla, CA) and clones were subjected to di-deoxy DNA sequencing (373A Sequencing System, Perkin Elmer/Applied Biosystems, Foster City, CA).

Results

IL15 FORMS DISTINCT CLONAL EXPANSIONS IN PERIPHERAL BLOOD LYMPHOCYTES

We first examined whether IL15 could induce expansion of peripheral T cell clones *in vitro*, as the repertoire within a given T cell population that responds to IL15 is unknown at present. For this purpose, peripheral blood lymphocytes (PBL) were cultured *in vitro* in the presence of IL15, and the clonality of resultant stimulated T cells was analysed by RT-PCR-SSCP. Figure 1A shows representative results of the final SSCP. Based on our previous studies, expanding T cell clones appear as a band on SSCP films in contrast with the highly heterogeneous background population, which show a broad smear-like pattern.³ As shown in fig 1A, precultured PBL from healthy subjects and patients with RA mostly showed a smear pattern (that is, a heterogeneous population), consistent with the results of previous studies from our laboratory.³⁻⁵ On the other hand, IL15 stimulated cells showed a distinct pattern with bands, indicating oligoclonal T cell expansion induced during culture.

Figure 1B shows the results of SSCP in a representative patient with RA. The results of precultured PBL again showed almost a smear pattern (fig 1B, PBL). In contrast, SF from the same patient showed distinct clonal T cell accumulations in almost all BV families studied (fig 1B, SF). These results are also in agreement with our previous findings.³⁻⁵

Next, we analysed cytokine stimulated PBL. As shown in fig 1B, stimulation with IL2 or IL15 induced clonal expansion of particular T cell clonotypes from the heterogeneous group of PBL. On the other hand, stimulation with phytohaemagglutinin (PHA) did not produce new clonal expansions of T cell clonotypes (fig 1B). Cultures prepared without the addition of cytokine showed cell death (data not shown).

Taken together, the above results indicated that IL15, like IL2, could induce clonal expansion of T cells from an otherwise heterogeneous peripheral population of PBL.

IL15 AND IL2 STIMULATION INDUCED DIFFERENT CLONAL EXPANSIONS

As shown in fig 1B, IL2 and IL15 were found to induce distinct patterns of clonal expansion. As IL15 and IL2 are known to share a common IL2 receptor and similar biological activities, we compared the T cell clonality induced by IL15 and IL2. To this end, we electrophoresed the samples of stimulated cells onto the same SSCP gel simultaneously and compared the T cell clones with each other. We have previously confirmed that clones that migrate to the same position of the same SSCP gel are identical clones with identical T cell receptor (TCR) CDR3 sequences.^{4,5}

Table 1 Sequence analyses of T cell clonality of in vitro stimulated and in vivo accumulating T cells in a representative patient with rheumatoid arthritis. Underlined clones are common in IL15 and IL2 stimulated T cells

Patient No3, BV2				
SF				
CSA	RDQQSF	SGNTI	1.3	12/31
CSA	RLTRDVPH	EQFFG	2.1	2
CSA	GPRDKEY	YEQYF	2.7	2
CSA	PDRGPT	SYEQY	2.7	2
CSA	RDHGGLS	SYEQY	2.7	2
CSA	RDPTSSH	NEQFF	2.1	1
CSA	RESUTSLN	EQYFG	2.7	1
CSA	RFSGG	YGYTF	2.7	1
CSA	GTGTGAP	DTQYF	2.3	1
CSA	ASGGL	YNEQE	2.1	1
CSA	DEPDH	YEQYF	2.7	1
CSA	LAGG	YEQYF	2.7	1
CSA	RAGGT	YNEQF	2.7	1
CSA	RDFSGRD	YEQYF	2.7	1
CSA	RARVTD	EQYFG	2.7	1
CSA	REE	ETQYF	2.5	1
IL15				
CSA	REGGT	YNEQF	2.1	2/17
CSA	RVA	GELFF	2.2	1
CSA	DPGQP	TGELF	2.2	1
CSA	GKPHGP	YEQYF	2.7	1
CSA	VPGTAS	SYNEQ	2.1	1
CSA	REGA	NTEAF	1.1	1
CSA	RKTGS	PQHFG	1.5	1
CSA	RRAGGH	YEQYF	2.7	1
CSA	RPPSGRAH	NEQFF	2.1	1
<u>CSA</u>	<u>SSQGKG</u>	<u>YFGPG</u>	2.7	1
CSA	RGGI	NYGYT	1.2	1
C	SPVVGPIT	YEQYF	2.7	1
CSA	RRAGHSN	YEQYF	2.7	1
CSA	RERLDRSF	YEQYF	2.7	1
CSA	RDRQY	SNQPP	1.5	1
CSA	RDMGQT	YEQYF	2.7	1
IL2				
<u>CSA</u>	<u>SSQGKG</u>	<u>YFGPG</u>	2.7	4/21
CSA	PTGGGN	SYEQY	2.7	4
CSA	NGIQGD	SPLHF	1.6	3
CSA	PTGGN	SYEQY	2.7	1
CSA	KQATKNL	QYFGR	2.7	1
CSA	NRGL	SYEQY	2.7	1
CSA	RDRGS	YEQYF	2.7	1
CSA	KRGRDS	SYEQY	2.7	1
CSA	RVA	GELFF	2.2	1
CSA	AP	YNEQF	2.1	1
CSA	RDRQGVR	TEAFF	1.1	1
CSA	TGWTGGN	EQYFG	2.7	1
CSA	PPSLG	TGELF	2.2	1
CSA	GFGP	ETQYF	2.5	1
CSA	RRFRLAGDR	YEGYF	2.7	1
CSA	TQGN	GANVL	2.6	1
CSA	QGGGI	YEQYF	2.7	1
Patient No 3, BV 18				
SF				
CASSP	SGAPG	SGNTI	1.3	4/25
CASS	GEVVD	EQFFG	2.1	2
CASS	PPGNT	YEQYF	2.7	2
CAS	SLLATP	YEQYF	2.7	1
CASS	FRAK	SYEQY	2.7	1
CASSP	EA	YNEQF	2.1	1
CASS	PDGQG	NTGEL	2.2	1
CASS	QGP	NTGEL	2.2	1
CASS	SGTG GTLG	GYTFG	1.2	1
CASS	RGTVSTDT	QYFGP	2.3	1
CASS	PDMGTN	NEQFF	2.1	1
CASS	PGQAEGT	NEQFF	2.1	1
CASSP	PTGM	DTQYF	2.3	1
CASS	PVRDV	QFFGP	2.1	1
CAS	SAGFVD	EQFFG	2.1	1
CAS	SSGV	SNQPP	1.5	1
CASSP	LKGR	ETQYF	2.5	1
CASS	PWQVEGF	NEQFF	2.1	1
CASS	PTG	YNEQF	2.1	1
CASS	RGTV	STDTQ	2.3	1
IL15				
<u>CASS</u>	<u>PVTSGG</u>	<u>YEQYF</u>	2.7	6/20
CASS	HRTGGS	EQFFG	2.1	5
CASS	PRLGG	GELFF	2.2	3
CASS	PTIN	TDTQY	2.3	3
CASS	VQGAH	TEAFF	1.1	1
CASSP	HE	QETQY	2.5	1
CASS	PQRGLGT	ETQYF	2.5	1

Table 1 Continued

Patient No3, BV18				
IL2				
CASSP	ILGS	YNEQF	2.1	4/21
<u>CASS</u>	<u>PVTSGG</u>	<u>YEQYF</u>	2.7	3
CAS	SSGQGAMV	EQFFG	2.1	1
CAS	QNM	DTQYF	2.3	1
CASS	PGN	QETQY	2.5	1
CAS	STNLAGG	TGELF	2.2	1
CASS	WGLS	SYNEQ	2.1	1
CASS	PVTSGD	EQFFG	2.1	1
CASS	PALAGPS	NEQFF	2.1	1
CASS	PLRT	QETQY	2.5	1
CAS	SSKSGTS	NTQEL	2.2	1
CASSP	QRDF	GNTIY	1.3	1
CASS	LSGSF	TGELF	2.2	1
CASSP	PSLG	SYNEQ	2.1	1
CASS	PFSG	NGEQF	2.1	1
CAS	STNLAGG	TGELF	2.2	1

Figure 2 shows the results obtained. The accumulating clones induced by IL15 were found to be partly, but not completely, identical with those induced by IL2 (lanes 2 and 3). For example, in BV3+ T cells from patient 1, among six dominant clones that were induced by IL15, only one clone migrated to a position identical with that of one of the three IL2 induced clones. In contrast, in the BV14+ T cell population in the same patient, three out of four IL15 induced clones were identical with the respective three clones expanded by IL2. In general, approximately 1/6 (17%) to 3/4 (75%) IL15 induced T cell clones were identical with the IL2 responding clones in this patient with RA. Conversely, we found that 1/5 (20%) to 3/3 (100%) clones induced by IL2 were identical with IL15 induced clones in the same patient. These results indicated that the IL15 responding T cell clones were not totally identical with the IL2 responding population.

IL15 INDUCED PERIPHERAL BLOOD T CELL CLONAL EXPANSION WAS NOT IDENTICAL WITH THAT ACCUMULATING IN RA JOINTS

As shown also in figs 1B and 2, T cell clonal expansion was identified in joints of patients with RA in vivo. To test whether IL15 plays a part in T cell clonal expansion in the joints we compared the clonality of T cells present in SF with that of IL15 stimulated PBL in each patient. Results of SSCP analysis showed that most expanding peripheral clones induced by IL15, as well as those induced by IL2, were not identical with the in vivo accumulating clones in SF (fig 2, lanes 2, 3, and 4). Although there were identical clones that were commonly activated by IL15 and by IL2, none of these clones was found in the SF (for example, fig 2, patient 3, BV14). These results indicated that most clonally accumulating T cells in SF were unlikely to be induced by IL15.

To provide quantitative confirmation of the results of the above SSCP analysis, we performed sequence analysis of TCR CDR3 regions of each T cell population. Specifically, we randomly cloned TCR genes derived from stimulated T cells as well as in vivo accumulating cells in SF and subsequently analysed the gene sequences of these cells. Table 1 summarises representative results of analyses of BV2+ and BV18+ T cells. It was confirmed that T cells of SF contained oligoclonally expanding

T cells, as shown by SSCP (table 1, SF and fig 1B, SF). On the other hand, stimulation by IL15 or IL2 did form clonal expansions in peripheral T cells (table 1, IL15 and IL2). When compared with each other, a proportion of IL15 induced clones were identical with IL2 induced clones (for example, clone CASS-PVTSGG-YEQYF in BV18, table 1, underlined). However, none of the IL15 induced clones was identical with the in vivo accumulating clones in synovial lesions (table 1, IL15 and SF). Similarly, none of IL2 induced clones was identical with the SF clones. These results, together with those of sequence analysis, suggested that the in vitro effects of IL15, and those of IL2, could not reproduce the in vivo T cell clonalities of RA joints.

Discussion

McInnes and Liew have recently suggested that IL15 could recruit and expand CD45RO+ memory T cell subsets in the synovial membrane and that such non-specific activation could perpetuate the inflammatory process through the induction of synthesis of monocyte derived TNF α .⁹ To confirm this hypothesis the clonality of synovial accumulating T cell clones should be identical with that of peripheral IL15-responsive T cells. In this study we investigated this using RT-PCR-SSCP and sequencing methods. Our results showed inconsistency between in vivo T cell clonality and IL15 induced clonality, suggesting the existence of a distinct immune response other than non-specific T cell recruitment in the affected joints of RA.

IL15 is reported preferentially to activate "memory" T cells that have recently encountered exogenous or endogenous antigens in vivo.^{9,16} Therefore, the T cell clonotypes expanded by IL15 would reflect the antigen exposed population in peripheral blood. However, the accumulating T cell clones in SF in vivo were different from those induced by IL15 in peripheral blood. Thus it is likely that synovial T cells do not expand in response to IL15 only, but rather to other factor(s), including antigenic stimulation within the joint. In fact, our previous study showed that the major component of accumulating T cell clones in SF was of the persistent type.⁵ In another study from our laboratory we also showed the recognition of type II collagen by at least some

synovial accumulating T cells.¹⁴ Thus most joint accumulating T cell clones are distinct from the non-specifically activated or recruited population in the periphery. Specifically, intra-articular T cell clones probably expand in response to various intra-articular antigens, such as type II collagen, acting as an autoantigen. Although it is difficult to determine the antigen specificity of IL15 responsive clones in the periphery, they may represent a "reservoir" of rather systemic immunological memory.

It should be emphasised, however, that our results do not exclude the role of IL15 in the pathogenesis of RA. As recent reports have suggested, IL15 can induce chemokine expression,¹⁷ adhesion receptor redistribution,¹⁸ and transendothelial migration^{19,20} of T cells. These IL15 effects, in addition to the direct effect on T cell growth, would enhance the inflammatory process in RA. In fact, Ruchatz *et al* reported the prevention of collagen induced arthritis, a murine model of RA, by an IL15 antagonist.²¹

Differences in the response to IL15 and IL2 of a given T cell population are not fully understood. In this study we showed that these two closely related cytokines induced accumulation of different clones, except for a small proportion of common clones from diverse peripheral T cells. This difference may be explained by differential expression of IL15 receptor or IL2 receptor on each T cell clone. Thus Kanegane and Tosato reported differences in IL2 receptor expression and response to IL2 and IL15 between naive or memory subsets of CD4+ cells.¹⁶ In addition, recent reports suggest different effects of IL2 and IL15 on effector generation of CD4+²² and CD8+²³ T cells. Moreover, IL2, but not IL15, might induce upregulation of Fas ligand expression and downregulation of common cytokine receptor γ chain expression.²⁴ Such observations indicate that each T cell clone within each phenotypic population might express a different pattern of the cytokine receptor, which might result in a different sensitivity to the cytokines. More detailed analysis of the differences in IL2 receptor or IL15 receptor expression, or both, or differences in antigen specificity between IL15 receptor(+) and IL2 receptor(+) cells, is therefore necessary to clarify the in vivo effect of IL15.

In vitro stimulation of T cells by IL15 induced clonal expansion not only in patients with RA but also in healthy subjects. The frequency of the responding T cell clones was similar between the two groups of subjects. This finding implied that the difference in frequency of already sensitised, "antigen experienced" T cells in the peripheral circulation is small between normal subjects and patients with RA. This is probably because RA is an organ-specific autoimmune disease in which the antigenic exposure of T cells is likely to be limited to within the affected joints. The degree of T cell proliferation, measured by [³H]thymidine incorporation, was the same in two normal controls and the patients with RA (data not shown).

In this study we used IL15 at 100 ng/ml for in vitro studies, similar to the protocol used in another study by Sebbag *et al*.²⁵ At this concentration, IL15 stimulates peripheral blood T cells to induce monocytic TNF α production.^{12,25} Possibly, the in vivo concentration of IL15 is much lower than this level, and the high concentration used in vitro might have influenced the resultant T cell clonality in this experimental system. However, as the number of expressed cytokine receptors per cell is limited, the stimulatory signal acting through the receptor might have reached a plateau. To test this conclusion it is important to investigate T cell clonality using different concentrations of IL15. It has been reported, however, that local synovial fluid IL15 concentrations do not correlate with various parameters of systemic disease activity or with the response to drug treatment in patients with RA.¹⁰

In conclusion, we showed in this study that although IL15 may induce T cell clonal expansion in vitro, it does not contribute to T cell clonality in the synovial fluid of RA. However, IL15 may still modulate the pathological process in RA joints because it mediates the production of TNF α by macrophages and synovial T cells.¹²

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